NUCLEOCYTOPLASMIC TRANSPORT OF MACROMOLECULES THROUGH THE NUCLEAR PORES OF *XENOPUS* OOCYTES

By

STEVEN IRA DWORETZKY

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1988

DEDICATED TO MY GRANDPARENTS, LEON AND FRANCES DWORETZKY

ACKNOWLEDGEMENTS

The studies presented in this dissertation could not have been performed without the guidance and assistance of Dr. Carl Feldherr. He served as an excellent role model for conducting scientific research of the highest caliber. I would like to express my gratitude to him for allowing me to work in his laboratory throughout the past three years. Special thanks are extended to Dr. Robert Cohen for serving as a committee member and providing insight to the biophysical aspects associated with my research. I would like to thank Drs. C. West and G. Stein for serving as members of my committee and providing critical review of the manuscripts submitted for publication.

I am grateful to Dr. Robert Lanford at the Southwest Foundation for Biomedical Research in San Antonio, Texas, for providing all of the BSA-conjugates that were used to study the effects of signal number on protein uptake.

The work presented in Chapter II is reproduced from the Journal of Cell Biology, 1988, volume 106, number 3, pages 575-584 by copyright permission of The Rockefeller University Press.

I would like to thank Denifield Player for advice concerning techniques in electron microscopy and Linda Mobley for typing of manuscripts and other pertinent material throughout my graduate career.

Finally, and most importantly, I wish to express my utmost gratitude to Barbara Ludwig. She was a constant source of moral support and encouragement throughout the trials and tribulations of my graduate education. In addition, I would like to thank my parents for their words of encouragement and steadfast support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

α	Alpha
Å	Angstrom
ATP	Adenosine-5'-triphosphate
Arg	Arginine
β	Beta
BSA	Bovine serum albumin
cT	Mutant large T-antigen
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
G	Guanosine
^{3}H	Tritiated
h	Hour(s)
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfuric acid
^{125}I	Iodinated
IgG	Immunoglobulin
kd	Kilodaltons
M	Molar concentration
min	Minute(s)
ml	Milliliter(s)
mM	Millimolar

mol wt	Molecular weight
N	Normal concentration
N/C	Nuclear to Cytoplasmic
ng	Nanogram
nl	Nanoliter(s)
NTPase	Nucleotide triphosphatase
OsO_4	Osmium tetroxide
p	Probability
PAGE	Polyacrylamide gel electrophoresis
Poly(A)	Polyadenylic acid
Poly d(A)	Poly-deoxy-adenylic acid
Poly(I)	Polyinosinic acid
Poly(U)	Polyuridylic acid
Pro	Proline
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
hnRNA	Heteronuclear ribonucleic acid
mRNA	Messenger ribonucleic acid
rRNA	Ribosomal ribonucleic acid
tRNA	Transfer ribonucleic acid
RNP	Ribonucleoprotein
s	Second(s)
SDS	Sodium dodecyl sulfate
SV 40	Simian virus 40
T-antigen	Tumor antigen
U	Uridine

µl	Microliter(s)
µm	Micron(s)
Val	Valine
vol/vol	Volume/Volume
WGA	Wheat germ agglutinin
WT	Wild type large T-antigen
wt/vol	Weight/Volume

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

NUCLEOCYTOPLASMIC TRANSPORT OF MACROMOLECULES THROUGH THE NUCLEAR PORES OF XENOPUS OOCYTES

By

STEVEN IRA DWORETZKY

August 1988

Chairman: Carl M. Feldherr
Major Department: Anatomy and Cell Biology

To localize and characterize the pathways for RNA translocation to the cytoplasm, gold particles coated with tRNA, 5S RNA, or poly(A) were microinjected into the nuclei of Xenopus oocytes. At various times after injection, cells were fixed and the particle distributions analyzed by electron microscopy. Similar results were obtained with all RNAs used. Particles ranging from 20-230 Å in diameter were observed within central channels of the nuclear pores and in the adjacent cytoplasm. Particles larger than 90 Å would not be expected to diffuse readily through the pores, suggesting that mediated transport occurred. Furthermore, approximately 97% of the pores analyzed were involved in the translocation of gold particles. After nuclear injection, particles coated with exogenous macromolecules were essentially excluded from the

pores and cytoplasm. These results indicate that translocation of RNA-coated gold was due to the presence of RNA associated with the particles.

To determine if the number of targeting signals affects the transport of proteins into the nucleus, oocytes were injected with gold particles coated with BSA cross-linked with various numbers of short synthetic peptides containing the SV 40 large T-antigen nuclear transport signal. Large T-antigen also was used as a coating agent. Particles carrying active targeting signals entered the nucleus through central channels within the nuclear pores. Analysis of the intracellular distribution and size of the tracers entering the nucleus indicated that the number of signals per molecule affects both the uptake of particles and the functional size of the channels available for translocation. In control experiments, particles coated with BSA or BSA conjugated with inactive SV 40 transport signals were virtually excluded from the nucleus. Nucleoplasmin-coated gold was transported through the nuclear pores more effectively than the BSA conjugates. The accumulation of tracers along the nuclear envelope suggests that differences in the activity of the two targeting signals might be related to their binding affinity for envelope receptors.

Double injection experiments demonstrated that individual pores are capable of transporting both protein and RNA and they can also recognize and transport proteins containing different nuclear targeting signals.

CHAPTER I
A REVIEW OF NUCLEAR ENVELOPE PERMEABILITY: DIFFUSION AND TRANSPORT

Introduction

The formation of the nuclear envelope around the cell's genetic material is one of the major evolutionary differences between prokaryotes and eukaryotes. The nucleus, site of both RNA and DNA synthesis, contains the genetic information needed for phenotypic expression. The cytoplasm, on the other hand, is the site of protein synthesis, which requires certain RNAs that are exported from the nucleus. Furthermore, the nucleus is dependent on proteins synthesized in the cytoplasm. Therefore, cell growth, division, differentiation, and maintenance require a continual exchange of information between both the cytoplasm and nucleus. To better understand cellular function, it is important to determine the factors that control the rate of movement between the two compartments and the types of molecules that are exchanged. For this reason, knowledge of the nuclear envelope's structure and permeability properties is essential in understanding the interrelationships that exist between the nucleus and cytoplasm of the eukaryotic cell.

In the first part of this chapter an ultrastructural description of the nuclear envelope is provided, followed by a review of permeability experiments which have helped to elucidate the role of the envelope in regulating nucleocytoplasmic exchanges.

Morphology

The discovery of the nucleus is documented as early as 1833. Fifty years later, light microscopists began speculating that a membrane surrounded the nucleus. It was Chambers and Fell (1931) who microdissected cells and demonstrated the existence of a nuclear envelope. With the use of the electron microscope, Callan and Tomlin (1950) performed ultrastructural studies on manually isolated oocyte nuclei and confirmed the existence of an envelope surrounding the nucleus. They described it as a double membrane structure, with a porous outer membrane adjacent to the cytoplasm and a continuous inner membrane adjacent to the nucleoplasm.

With advances in the resolution of the electron microscope and improvements in fixation procedures, the nuclear envelope and pore structure became the subject of intense morphological studies. The envelope and associated structures can be visualized with the electron microscope by a variety of techniques including i) *in situ* fixation followed by ultrathin sectioning, ii) negative staining of isolated envelopes, iii) freeze-etching, and iv) high-resolution scanning microscopy. One of the first detailed studies utilizing transmission electron microscopy was performed by Watson (1955, 1959). He analyzed nuclei from a wide variety of cell types and found that the inner and outer membranes fuse at regular intervals along the envelope resulting in the formation of nonmembranous regions ("holes"), ranging from 800-1200 Å in diameter, referred to as the nuclear pores. These pores form channels that represent potential sites of interaction between the nucleoplasm and cytoplasm. Since then, laboratories have used the

procedures listed above to accumulate a vast amount of ultrastructural information concerning both the nuclear envelope and pore structure from a range of cell types, resulting in many interpretations of the pore's three-dimensional architecture. Comprehensive reviews of the morphology of the nuclear envelope and pore complexes have been published by Franke (1974), Harris (1978), and Maul (1977). The following discussion will be limited to a general morphological description of the nuclear envelope and its associated pore complex, without describing some of the finer substructures, since their existence remains controversial.

The nuclear envelope is considered to have four structural components: the inner and outer membranes, the pore complexes, and the nuclear lamina. The envelope, seen in thin sections perpendicular to the nucleus, is composed of a double membrane system with the inner and outer membrane parallel to one another until they approach the pore complexes. In general, each membrane is approximately 75 Å in diameter. A homogenous region, which varies in width from 100-600 Å depending on the cell type, lies between the two membranes and is referred to as the perinuclear cisterna (Wischnitzer, 1958). Often, the luminal space of the perinuclear cisterna is observed to be in direct continuity with the luminal cavity of the endoplasmic reticulum (Watson, 1955). The outer and inner membranes are associated with different structures. Polyribosomes are seen to be attached to the outer membrane, whereas, the inner surface of the inner membrane appears to have an intimate association with a distinct morphological layer, referred to as the lamina. These observations suggest that the inner and outer membranes

are different, although they have not been analyzed by biochemical methods.

As visualized by electron microscopy, the nuclear lamina is a discrete layer of material, varying in thickness from 30-100 nm, lining the entire inner surface of the nuclear envelope, excluding the pore complexes. A lamina enriched fraction is obtained by non-ionic detergent and high salt extraction of isolated envelopes (Aaronsen and Blobel, 1975; Dwyer and Blobel, 1976). Protein analysis of the residual fraction shows the presence of three predominant proteins, referred to as lamins A, B, and C. They comprise approximately 40% of this fraction. During interphase, these polypeptides have been localized, by immunocytochemistry, exclusively to the lamina at the nuclear periphery (Gerace et al., 1978). Gerace and Blobel (1982) have shown that the lamina forms a fibrous network that is a component of the nuclear skeleton. The lamina has been implicated to function as a structural framework for the nuclear envelope and an anchoring site at the nuclear periphery for chromatin. In addition to its structural role, the lamina is thought to be involved in reversible nuclear envelope assembly and disassembly during mitosis (Gerace et al., 1984).

The pores are filled with diffuse electron-opaque material that extends a short distance into both the nucleus and cytoplasm. In tangential sections, this electron-opaque material has a ring-like appearance and has been referred to as the annular material (Watson, 1955; Wischnitzer, 1958). The pore with its associated annulus is called the pore complex.

Discrete subunits, 100-250 Å in diameter, are distributed evenly around both the cytoplasmic and nuclear margins of the pore (Watson, 1955; Wischnitzer, 1958). Franke (1966) using a multiple rotational exposure technique on negative-stained preparations of isolated envelopes, describes the arrangement of annular globules (granules) to be in eight-point radial symmetry. Furthermore, the subunits lying on the pore margin within the nucleus overlap the same sites as subunits on the cytoplasmic pore margin.

Within the centers of some pores, an electron-dense granule with diameters ranging from 40-350 Å can be observed. This central granule has been visualized with different preparative techniques; however, the observed frequency of its presence is highly variable (Franke and Scheer, 1970). It has been suggested that the granule is actually material in transit, e.g. ribonucleoprotein (RNP), and that a correlation exists between the frequency of the central granule and the activity of RNA synthesis (Scheer, 1970). Cytochemical studies performed by Franke and Falk (1970) support this view. Even with this data it still remains controversial whether the granule is a permanent structural component of the pore complex or material passing through the pore.

Thin filaments, 25-50 Å in diameter, have also been observed in the interior of the pore. These filaments radiate laterally from the pore margins and appear to connect with the central granule (Franke, 1974). The filaments have a spokelike radial appearance and exhibit eightfold symmetry similar to that of the annular globules.

Unwin and Milligan (1982) have used thin section electron microscopy coupled with Fourier averaging methods to describe the pore's three-dimensional organization to a resolution of 90 Å. They found the pores, in Xenopus oocytes, to occupy 20-30 percent of the envelope surface and are composed of several discrete components: plugs, spokes, particles, and rings. The rings have internal and external diameters of 800 Å and 1200 Å, respectively. Spokes extend radially from the margins of the channel toward a plug (30-350 Å in diameter) located in the center of the pore. Particles are seen on the cytoplasmic surface of the pores but do not appear to be integral components, as they can be released with high salt treatments. This model, with its high resolution of analysis, confirms many of the components put forth in the model described by Franke (1974).

Until recently, little was known about the molecular composition of the pore complex. Gerace et al. (1982) have identified a 190 kd intrinsic membrane glycoprotein that is specifically localized to the nuclear pores in liver nuclei. After detergent treatment, which solubilizes most nuclear envelope intrinsic proteins, the 190 kd glycoprotein remains associated with the pore complex-nuclear lamina fraction. Based on this and its biochemical properties, it is suggested that the 190 kd glycoprotein is involved in anchoring the pore complex to the envelope. Davis and Blobel (1986), using monoclonal antibodies and immunoelectron microscopy, have identified a 62 kd protein that localizes within the annuli of rat liver nuclei. Snow et al. (1987) have described a group of eight glycoproteins, with common epitopes, that are localized exclusively to the nuclear pore complex. The group

of eight glycoproteins exist in multiple copies per pore complex and their function is unknown at this time.

Compiling the data available on the components of the pore complex, many investigators have suggested models or schematic representations of the nuclear envelope architecture. These models, in conjunction with the biochemical data, provide a framework for investigating how the nuclear pores might be involved in regulating nucleocytoplasmic exchanges. The appendix presents a schematic that is based on Franke's (1974) and Unwin and Milligan's (1982) data.

Nuclear Permeability Experiments

It is clear from morphological evidence that the nuclear envelope with its associated pore complexes is different from the plasma membrane and other intracellular membranes. Since cellular processes both within the nucleus and cytoplasm are dependent on a mutual exchange of material, an important question arises as to the permeability properties of the nuclear envelope and to what extent the envelope might regulate nucleocytoplasmic exchange.

Three general approaches are used to study intracellular exchanges across the nuclear envelope. First, microinjection of labelled tracers (fluorescent, radioactive or electron-dense) is employed extensively to study the permeability of the nuclear envelope to molecules within intact cells. Second, permeability experiments can be performed on isolated nuclei; however, these results must be carefully interpreted as the permeability characteristics can change as a result of the isolation (e.g., Paine et al., 1983; Peters, 1986). Third, recombinant DNA

methodology has been used to probe structural features of proteins that are specifically targeted to the nucleus.

Ion permeability

Ions are known to interact with chromatin and activate specific genes. During mitosis, meiosis, DNA synthesis, and hormonal stimulation, ionic changes are occurring within the cell; thus, it is important to understand intracellular ion distributions and how the envelope might regulate their exchange between the nucleus and cytoplasm.

Earlier permeability studies were qualitative in nature and based on the structural or colloidal changes that occurred within isolated nuclei following incubation in different salt solutions. It was concluded from these studies that both cations and anions can rapidly penetrate the nuclear envelope.

The use of radioactive isotopes as tracer molecules provided a new method to give precise data on the permeability of the envelope. Abelson and Duryee (1949) incubated individual oocytes in $^{24}Na^{+}$ and later studied the intracellular ion distribution by quick freezing and radioautography. The results demonstrated that the nuclear envelope in amphibian oocytes was readily permeable to $^{24}Na^{+}$. In addition to these findings, the nucleus, at equilibrium, contained twice as much radioactive tracer per unit volume as the cytoplasm. The measurement of water content in both compartments indicated the nucleus to have a higher percentage of water than the cytoplasm, which could account for the difference in Na^{+} concentrations. Other molecules, $^{42}K^{+}$, $^{32}PO_4^{=}$,

$^{35}SO^{=}_{4}$, leucine-^{14}C, and alanine-^{14}C were shown by Naora et al. (1962) to pass readily across the nuclear envelope.

To measure precisely the intracellular distribution of Na^{+}, Horowitz and Fenichel (1970) utilized ultra-low temperature autoradiographic techniques. They described at least three intracellular Na^{+} fractions: a cytoplasmic fast fraction, a cytoplasmic slow fraction, and a nuclear fast fraction. The fast fractions were thought to represent freely diffusible Na^{+}, whereas the slow fraction is interpreted to be Na^{+} bound to nondiffusible elements within the cytoplasm. This slow diffusion fraction could account for the higher concentration of the Na^{+} ion found within the cytoplasm. Therefore, the asymmetric ion concentrations can be accounted for by two factors: first, the differences in water content between the nucleus and cytoplasm, and second, possible binding in the cytoplasm.

Another method for studying the permeability of the nuclear membrane to ions is by measuring the envelope's electrical resistance. Inserting intracellular microelectrodes into the nucleus and cytoplasm of amphibian oocytes, Kanno and Loewenstein (1963) found no appreciable resistance, indicating that ions are able to diffuse freely across the envelope. Comparing the results from oocytes, the flux measurements and electrical resistance data are in good agreement.

The results presented above suggest that the nuclear envelope is not a barrier against ion diffusion. Since the nuclear envelope does not impede ion passage and ion transport by the nuclear envelope is not indicated (Century et al., 1970), it is likely that ions pass through the aqueous channel formed by the nuclear pores.

Small molecules

Permeability of the nuclear envelope to small molecules was studied by microinjecting sucrose (Horowitz, 1972), inulin (Horowitz and Moore, 1974), and the non-metabolizable amino acid α-aminoisobutyric acid (AIB) (Frank and Horowitz, 1975) into oocytes. All of these small molecules rapidly crossed the envelope such that standard flux measurements could not accurately define the rate of movement, suggesting that the nuclear envelope is of equivalent permeability to an equal thickness of cytoplasm. To better define the uptake kinetics of small molecules, Kohen et al. (1971) used high resolution microfluorimetry to quantitate rapid movement across the envelope. They investigated the permeability of glycolytic intermediates. Their results demonstrate that the metabolites move across the nuclear envelope with a 35 millisecond delay, indicating that for small molecules the nuclear envelope does not constitute a permeability barrier. Thus, similar to ions, it is likely that small molecules migrate into the nucleus through the nuclear pores.

Exogenous macromolecules

The nuclear uptake of radio-labelled or fluorescein-labelled exogenous molecules has been investigated by microinjecting these tracers into the cytoplasm of cells and following their intracellular distribution with time. Colloidal gold particles and ferritin, both of which can be visualized with the electron microscope, have also been used to study nuclear permeability. Microinjection of these exogenous tracers provides a method to better understand some of the physical characteristics of the nuclear envelope with respect to diffusion.

The pores, from a morphological standpoint, would appear to be a likely pathway for movement into and out of the nucleus. In support of this view, Feldherr (1962) injected colloidal gold particles coated with polyvinylpyrrolidone (PVP) into the cytoplasm of the amoebae Chaos chaos and localized the distribution of the tracers by electron microscopy. After 1-2 minutes, gold particles were observed in the centers of the pores and 24 hours after injection, gold particles were found to accumulate in the nucleus. Injection of different size gold fractions, 20-50 Å and 20-160 Å in diameter, showed that small gold particles localized within the nucleus after 3 min, in contrast to the larger gold fraction which had negligible uptake after 10 min. These results indicate that the rate of movement across the nuclear envelope is inversely proportional to the molecular size of the tracer. After longer time intervals, it was determined for Amoeba proteus that PVP-coated gold particles up to 120 Å can penetrate the pores of the nuclear envelope, whereas in Chaos chaos, particles as large as 140 Å can enter the nucleus (Feldherr, 1965). It is important to note that the maximum size of the particles able to penetrate the envelope is smaller than the 800 Å internal diameter of the pores. Overall, it was concluded from these studies that pores can serve as pathways for nucleocytoplasmic exchange but can restrict the movement of large molecules since the entire area of the pore is not available for free exchange.

Gurdon (1970) studied permeability properties of the nuclear envelope by injecting ^{125}I-labelled histone (10,000-20,000 kd) and bovine serum albumin (67,500 kd), into amphibian oocytes. After 50 minutes, it was demonstrated by autoradiographic analysis that the

nucleus accumulated ^{125}I-histone to at least twice the concentration of the cytoplasm. In contrast, the ^{125}I-BSA was more concentrated in the cytoplasm, even after 12 hours. It was clear from these results that the nuclear envelope shows some selectivity for inhibiting passage of large exogenous proteins.

The nucleocytoplasmic exchange of a range of exogenous macromolecules was studied quantitatively by Paine and Feldherr (1972). Well characterized macromolecules with respect to size, shape, and electrical charge were used: ferritin (mol wt 465,000; pI 4.4), BSA (mol wt 67,000; pI 4.71), ovalbumin (mol wt 45,000; pI 4.7), myoglobin (mol wt 17,500; pI 6.9), lysozyme (mol wt 14,500; pI 11.8); and cytochrome c (mol wt 13,000; pI 10.7). All the proteins were fluorescently labelled, with the exception of ferritin which is electron-opaque, and injected into the cytoplasm of oocytes from Periplaneta americana. Their results showed that ferritin and BSA had extremely low N/C ratios even after 5 h. Ovalbumin slowly entered the nucleus; however, a higher concentration was present in the cytoplasm as compared to the nucleus after 5 h. On the other hand, cytochrome c entered the nucleus within 5 min, whereas lysozyme and myoglobin had N/C ratios greater than 1.0 within 3 min. These results demonstrated that neutrally or positively charged proteins, less than 20 Å in diameter, can readily enter the nucleus. Furthermore, the nuclear uptake of the proteins, 20-95 Å in diameter, was inversely related to size and not simply based on charge. Similar conclusions were reached by Bonner (1975a) who studied the nuclear uptake of the small basic proteins;

histones, lysozyme, and trypsin inhibitor, and the neutral protein, myoglobin.

To determine if exogenous macromolecules exit the nucleus, Paine (1975) injected ovalbumin, horseradish peroxidase, myoglobin, and cytochrome c directly into the nucleoplasm of Chironomus salivary gland cells. The results showed that the fluorescein-labelled molecules enter and leave the nucleus with similar kinetics, indicating that nucleocytoplasmic exchange is bidirectional and the rate of movement across the envelope is dependent on 1) the restrictions imposed by the diameter of the diffusion channel and 2) the size of the molecule.

To estimate the patent diameter of the nuclear pores in amphibian oocytes, Paine et al. (1975) injected tritiated dextrans with known hydrodynamic radii of 12.0, 23.3, and 35.5 Å. Dextrans are uncharged polymers of D-glycopyranose and available in a range of molecular sizes. To determine the intracellular localization of the tracers, the cells were quickly frozen at different time intervals, sectioned, and autoradiographed. Comparison of the grain profiles for all three tracers showed that the nuclear envelope is less permeable to larger dextrans, supporting the view that the envelope can limit the rate of entry of larger macromolecules. The relationship between the size of the dextrans and their rates of entry suggested that the nuclear envelope had a sieving effect due to its porous nature. Developing a mathematical model from the rates of uptake, diffusional coefficients, and the length of the channel, the authors estimated the patent pore diameter to be 90 Å.

Stacey and Allfrey (1984) also used well characterized proteins to study the permeability of the nuclear envelope of cultured cells. Thirteen proteins ranging from 13 kd to 669 kd with pI's from 4.4 to 11 were microinjected into the nucleus or cytoplasm of HeLa cells. Proteins less than 40 kd entered almost immediately, in contrast to proteins between 40 kd and 60 kd which entered more slowly. Many of the proteins larger than 60 kd were excluded from the nucleus. The rates of diffusion into and out of the nucleus were indistinguishable.

To estimate the patent pore diameter in cultured liver cells, Peters (1984) used fluorescent microphotolysis to study the permeability properties of the nuclear envelope. Fluorescein-labelled dextrans ranging from 3 to 150 kd were injected into hepatocytes and allowed to reach equilibrium. A pulse from an argon laser depleted the fluorescent signal within a region of the nucleus and then microfluorimetry was used to measure the influx of the tracer back into the depleted region. His results showed the influx of the dextrans, from the cytoplasm to the nucleus, to have an exclusion limit between 17 and 41 kd. Using similar equations as Paine et al. (1975), Peters derived the functional pore diameter to be 100-110 Å with respect to diffusion.

All of the experiments involving the microinjection of exogenous tracers support the view that the nuclear envelope has properties of a molecular sieve. The entry of exogenous tracers into the nucleus occurs by non-selective mechanisms, that is, diffusion through the pores. The rate of influx is inversely related to size which reflects some of the limitations imposed by the pore complex. The pore diameter has been estimated for amphibian oocytes (90 Å; Paine et al., 1975),

amoebae (120-140 Å, Feldherr, 1965), and hepatocytes (100-110 Å, Peters, 1984). These differences in size can markedly affect the exclusion limits and flux rates of macromolecules. For example, it would take approximately 1 h for a molecule, 50 Å in diameter, to diffuse through a pore with a diameter of 110 Å. In contrast, it would take the same molecule 8 h to pass through a pore with a diameter of 90 Å (estimates from Fig. 6; Paine et al., 1975). This can explain the 2-5 orders of magnitude difference in dextran flux rates between oocytes and hepatocytes.

Endogenous macromolecules

Subsequent nuclear permeability studies have focused on the selective uptake of endogenous proteins. Labelled endogenous proteins can serve as probes to investigate the normal molecular interactions and behavior found within cells, in contrast to exogenous molecules, which are unlikely to have intracellular interactions and solely used to characterize the passive permeability properties of the nuclear envelope. It soon became evident that the rates of nuclear accumulation of endogenous molecules were much greater than exogenous molecules of comparable sizes. The data demonstrated that exchanges of endogenous proteins between the nucleus and cytoplasm were controlled by selective processes, explaining in part, the observation that endogenous proteins distribute differently than exogenous tracers.

There are two possible mechanisms that can account for the rapid rate of endogenous protein accumulation within the nucleoplasm. First, proteins can diffuse freely through the nuclear pores and then can be

selectively retained within the nucleus, by binding to a nondiffusible substrate. Second, the exchange of some or all of the endogenous proteins, through the pores, can occur by facilitated uptake. This mode of exchange is governed by the intrinsic properties of the molecule. Following translocation across the envelope, accumulation in the nucleus can occur by selective binding or by another mechanism for irreversible transport.

It has been reported that many proteins accumulate in the nucleus against a concentration gradient--e.g., for example, histones (Gurdon, 1970) and N1/N2 (De Robertis et al., 1978)--attain nuclear to cytoplasmic ratios of 115 and 120, respectively. Austerberry and Paine (1982) used cryomicrodissection and two-dimensional electrophoresis to measure quantitatively the intracellular distribution of 90 proteins in the living cell. Their results demonstrated that at least 35 proteins within the nucleus have nuclear to cytoplasmic ratios greater than 10, which suggests some form of selective retention. Additional evidence to support nuclear binding comes from the experiments performed by Feldherr and Ogburn (1980). When the permeability properties of the envelope are altered, as shown by its failure to exclude labelled BSA, most of the endogenous nuclear proteins still accumulate within the nucleus (see below).

Bonner (1975b) studied the migration of *in vivo* labelled endogenous proteins into nuclei of amphibian oocytes. After injection of labelled nuclear proteins into the cytoplasm of unlabelled oocytes, the radioactivity was found to concentrate in the nucleus. If labelled cytoplasmic proteins were injected into the cytoplasm of unlabelled

oocytes, radioactivity was concentrated in the cytoplasm although some labelled protein entered the nucleus. From these results, Bonner suggested the existence of three classes of proteins: N proteins, found predominantly in the nucleus; C proteins, found predominantly in the cytoplasm; and B proteins, found in equivalent amounts in both the nucleus and cytoplasm. Feldherr (1975) measured the rate of nuclear uptake for endogenous proteins that were labelled with tritiated amino acids in vivo. His results demonstrated that newly synthesized nuclear proteins, ranging from 94,000 to 150,000 kd, were three times more concentrated within the nucleus as compared to the cytoplasm after 6 h. The rates of accumulation for endogenous proteins of this size were greater than expected in comparison to the results obtained for exogenous proteins of similar size.

To better understand the role of the nuclear envelope in regulating nucleocytoplasmic exchange of endogenous proteins, Feldherr and Pomerantz (1978) altered the diffusion barrier by using glass needles to puncture holes in the envelope and then measured the uptake of labelled nuclear proteins. The results, as determined by one-dimensional gel electrophoresis, showed no qualitative differences in the accumulation of nuclear proteins in punctured nuclei as compared to nuclei from nonpunctured control oocytes. Feldherr and Ogburn (1980) further studied the mechanism of selecting nuclear proteins by first disrupting the nuclear envelope and then using two-dimensional gel analysis, fluorography, and double-labelling techniques to determine intracellular distributions. Analysis of over 300 nuclear polypeptides showed less than five percent of the proteins varied between experimentally

punctured oocytes and nonpunctured controls. It was concluded from these results that nuclear binding plays a major role in regulating the nucleocytoplasmic distribution of endogenous proteins, and that the envelope does not function as a rate-limiting barrier.

It is apparent from the above studies that many of the nuclear proteins accumulate in the nucleus at significantly higher rates than exogenous molecules of similar size. As discussed previously, exogenous proteins larger than 45 kd (ovalbumin) do not readily enter the nucleus; however, endogenous proteins larger than 90 kd accumulate quite rapidly.

Evidence for facilitated transport was obtained by Feldherr et al. (1983) for RN1, a 148 kd nuclear protein, in the oocytes of Rana pipiens. After microinjection or in vivo labelling of RN1, the kinetic results demonstrated rapid accumulation within the nucleoplasm. Theoretical estimates of the diffusion rate of a 148 kd protein through the 90 Å channel indicated that the rate of uptake could not be accounted for by simple diffusion through the pores and subsequent binding. Thus, in this instance some form of mediated transport is apparently required for rapid accumulation.

Recent research has shown that signal sequences of some nuclear proteins are required to target macromolecules to the nucleus. Two approaches have been used to study the polypeptide domains that contain these sequences. The first approach is the partial digestion of a protein followed by analysis of the fragments capable of accumulating in the nucleus and the second approach employs DNA methodology.

The first approach has been successfully employed to study nucleoplasmin, a 110 kd karyophilic protein in Xenopus oocytes, which

rapidly accumulates in the nucleus. Nucleoplasmin is a thermostable, pentameric molecule present within the nucleus in a high concentration (Mills et al., 1980). It appears in the electron microscope as a disc with a molecular dimension of 74 Å in diameter (Earnshaw et al., 1980). Pepsin digestion selectively cleaves nucleoplasmin into two structural domains, a protease-resistant core and five protease-sensitive tails (Dingwall et al., 1982). Protease removal of the five tail domains leaves a pentameric core that is excluded from the nucleus after microinjection into the cytoplasm. In contrast, the tail domains, after cytoplasmic injection, are capable of nuclear accumulation. When the core is injected directly into the nucleus, it migrates throughout the nucleoplasm but is still excluded from the cytoplasm, indicating that the core has the ability to be retained by the nucleus but not the ability to migrate into the nucleus. These results demonstrate that the tail domain contains the necessary information to specify selective entry. By reducing the time of digestion, a mixture of pentameric cores that contain different numbers of tail regions can be prepared. A core molecule with only one tail is capable of entering the nucleus, but at one-tenth the rate of intact nucleoplasmin (Dingwall et al., 1982). Amino acid analysis of the tail domain (C-terminus) indicates an enrichment in lysine residues and a depletion in hydrophobic amino acids.

The second approach, utilizing genetic engineering, entails the construction of plasmids encoding proteins normally found in the cytoplasm linked to putative nuclear signal sequences. The end product is a hybrid protein, converting an otherwise cytoplasmic protein into a

protein localizing in the nucleus. After narrowing down a region of the protein necessary for nuclear localization, oligonucleotide-directed mutagenesis can be used to map out the essential amino acid sequence necessary for targeting. Overall, these approaches have been used to study an array of proteins that are targeted to specific organelles: endoplasmic reticulum, mitochondria, nuclei, and peroxisomes.

The simian virus (SV) 40 large T-antigen targeting signal has been the most extensively studied sequence that confers nuclear localization. Large T-antigen, during lytic infection, is responsible for the regulation of viral transcription and replication. The protein has 708 amino acids and in the monomeric form (can be tetrameric) has a molecular weight of 94 kd. The size of this protein suggests that it cannot enter the nucleus through the pores by simple diffusion, although large T-antigen is found predominantly within the nucleoplasm. Butel et al. (1969) described a large T-antigen from a mutant SV 40 hybrid virus that was localized in the cytoplasm of an infected cell. Comparison of the sequence analysis of wild type large T (WT) to the mutant large T-antigen (cT) revealed the amino acid at the 128 position was changed from lysine to asparagine (Lanford and Butel, 1984). The mutant large T-antigen was found to be soluble in the cytoplasm and capable of binding DNA (Lanford and Butel, 1980). Thus, the failure to accumulate in the nucleus was not caused by its inability to either diffuse throughout the cytoplasm or bind DNA, but caused by a mutation in a critical region of the protein sequence that is required for nuclear localization.

Kalderon et al. (1984a) used site-directed mutagenesis to identify a seven amino acid sequence within large T-antigen that is required for nuclear localization, with the lysine at position 128 being the critical residue. A change in this lysine residue to asparagine, threonine, isoleucine, leucine, methionine, or glutamine caused the large T-antigen to become localized in the cytoplasm of the cells. Mutations within the signal sequence on either side of the lysine influenced the rate of uptake and qualitative distribution of large T-antigen, but did not abolish transport activity. The minimum sequence required for nuclear localization is Pro-Lys-Lys^{128}-Lys-Arg-Lys-Val (Kalderon et al., 1984b). One important aspect of this signal sequence is its ability to function when removed from its normal environment and placed in a different region of a protein. For example, if the signal is linked to the amino terminus of either a mutated large T-antigen or chicken muscle pyruvate kinase (a nonnuclear protein), it can still confer nuclear localization. In addition, a synthetically synthesized signal peptide cross-linked to a carrier protein has the ability to target the conjugate into the nucleus of cultured cells (Lanford et al., 1986; Yoneda et al., 1987) as well as oocytes (Goldfarb et al., 1986). Thus, the functional autonomy of the targeting signal suggests that it does not require a fixed secondary structure.

Recently, nucleoplasmin has been cloned and sequenced (Burglin and De Robertis, 1987; Dingwall et al., 1987). The fifty amino acids from the carboxy terminal tail domain have two regions similar in homology with the nuclear localization signal from the SV 40 large T-antigen (Dingwall et al., 1987). In addition, two other regions have been

identified to have weak homology with targeting signals from the yeast MATα2 protein. It was postulated from these results that each tail domain might have four targeting signals, with a total of 20 signals per pentamer. Further experimentation on the tail domain has now demonstrated that each tail contains only 1 signal sequence consisting of approximately 14 amino acids and though it is similar to the SV 40 T-antigen targeting signal, it is not homologous (Dingwall, personal communication 1988).

The list of proteins containing nuclear targeting signals continues to be expanded. In a search to find a conserved sequence, several targeting signals have been compared to SV 40 large T-antigen. Protein signals that have similar amino acid sequences include nucleoplasmin (Dingwall et al., 1987), polyoma virus large T-antigen (Richardson et al., 1986), SV 40 VP1 (Wychowski et al., 1986), and Xenopus laevis N1/N2 protein (Kleinschmidt et al., 1986). Nuclear localization sequences from other proteins showing little homology to SV 40 large T-antigen include the adenovirus E1a protein (Lyons et al., 1987), the yeast proteins L3 (Moreland et al., 1985) and MATα2 (Hall et al., 1984), and GAL4 (Silver et al., 1984).

The translocation of proteins directly across intracellular membranes has been well documented for endoplasmic reticulum (Warren and Dobberstein, 1978) and mitochondria (Schatz and Butow, 1983). Proteins destined for either organelle contain a signal sequence, which is cleaved after entry. Protein import into the endoplasmic reticulum occurs by a cotranslational mechanism; however, this mechanism is unlikely for nuclear uptake since cytoplasmically injected proteins,

extracted from the nucleus, can accumulate back into the nucleus. Mitochondrial proteins have signal sequences that target them into specific regions of the mitochondria and cross the membrane(s) by a posttranslational mechanism. However, no evidence exists that nuclear transport occurs by a posttranslational process directly across the membrane, although it still remains a possibility.

An elegant approach to study the exchange sites of a known transportable nuclear protein was performed by coating colloidal gold particles with nucleoplasmin (Feldherr et al., 1984). Their results demonstrate that nucleoplasmin-coated gold enter the nucleus through channels located in the centers of the pores. Particles, ranging in size from 50-200 Å, were able to penetrate the pore region, thus indicating that the functional size of the transport channel is different than the size of the diffusion channel. The transported particles are nondeformable and spherical, which argues against structural deformation of nucleoplasmin prior to transport. Furthermore, the particles are markedly larger than the diffusion channel, which suggests the involvement of a selective uptake process.

In vitro uptake

The use of an in vitro assay system to investigate protein transport has both advantages and disadvantages. The drawbacks are, first, changes in the permeability characteristics of isolated nuclei. Peters (1983) measured the permeability of single isolated liver nuclei to dextrans using fluorescent microphotolysis and estimated the pore diameter to be 112-118 Å, which is 2-8 Å larger than pores from in vivo

nuclei (see exogenous molecules section). According to the model of Paine et al. (1975), a small change in pore diameter can have a marked effect on flux rates. Second, after isolation of oocyte nuclei under aqueous conditions, 95% of the proteins is lost with a half-time of 250 s (Paine et al., 1983). Third, assuming some nuclear proteins are transported along cytoskeletal elements to the nuclear surface, the isolation procedure removes the nucleus from its cytoplasmic anchorage, thus removing a possible step in transport. In light of these disadvantages, the results from the in vitro experiments must be carefully interpreted.

An in vitro system is advantageous to study protein uptake since the steps in the process can be easily dissected and experimentally manipulated. In vitro assays can be used: i) to characterize components needed for translocation, ii) to determine inhibitors of protein transport, and iii) to determine if protein transport is ATP-dependent.

Forbes et al. (1983) have shown that bacteriophage DNA microinjected into Xenopus eggs can form nucleus-like structures. Addition of DNA from either bacteriophage lambda or Xenopus to an extract from Xenopus eggs results in the formation of synthetic nuclei that have morphological similarities to intact nuclei (Newmeyer et al., 1986). These synthetic nuclei have a double membrane envelope, however, not all possess nuclear pore complexes. The reconstituted nuclei, in conjunction with labelled nucleoplasmin, have been used to investigate aspects of the protein transport process. Newmeyer et al. (1986) demonstrated that nucleoplasmin uptake is ATP- and temperature-

dependent. In addition, the lectin wheat germ agglutinin (WGA) effectively inhibits the transport of nucleoplasmin and the inhibitory effects could be blocked with the addition of N-acetylglucosamine (Finlay et al., 1987). Ferritin-labelled WGA was shown to be localized to the cytoplasmic face of the nuclear pores. Furthermore, iodinated WGA stains a glycoprotein, 63-65 kd, on nitrocellulose blots of rat liver nuclei (Finlay et al., 1987). This glycoprotein is similar to the one detected by Davis and Blobel (1986) and Snow et al. (1987). It has been suggested that this glycoprotein is part of the pore complex and might be involved in the nuclear transport of proteins.

Nuclear Efflux

Introduction

The nucleus synthesizes the RNAs required for protein synthesis; thus, the efflux of messenger RNA (mRNA), ribosomal RNA (rRNA), and transfer RNA (tNRA) is an obligatory step in the process of gene expression. Heterogeneous nuclear RNA (hnRNA), small nuclear RNAs (snRNA) and tRNA precursors are found normally within the nucleus. Prior to mature mRNA export to the cytoplasm, hnRNA must undergo i) processing, which involves removal of the introns and splicing together of the exons, and ii) posttranscriptional modifications, which can include polyadenylation and methylation. tRNA precursors must also be processed before efflux and precursor rRNA is processed into 28S, 18S, and 5.8S RNAs and are present in 40S and 60S RNP particles prior to transport into the cytoplasm.

Goldstein and Plaut (1955) obtained direct evidence in Amoeba proteus for the transfer of RNA to the cytoplasm by first labelling the RNA and then transplanting the labelled nucleus to an unlabelled recipient cell. As shown by autoradiography, the number of grains within the cytoplasm increased with time. In addition, no radioactivity was observed in the unlabelled recipient cell nucleus. Ribonuclease digestion was performed to show that the radioactivity was associated with the RNA. Due to the size of the RNA, it was postulated that simple diffusion was not the mechanism involved in RNA efflux.

The movement of RNA from nucleus to cytoplasm is likely to be a three-step process, involving 1) migration from its site of transcription to the inner surface of the nuclear envelope, 2) translocation across the envelope, presumably through the nuclear pores, and 3) movement from the outer surface of the nuclear envelope to its final destination within the cytoplasm. The first two steps have been the focus of intense investigation in an attempt to understand the mechanism(s) involved in RNA transport. The final step of directing RNA to its destination within the cytoplasm is not as well characterized as steps 1 and 2. In addition, Agutter (1985a) has suggested that RNA efflux occurs by a solid-state process; that is, movement occurs along structural elements both within the nucleus and cytoplasm.

Transfer RNA

The structure of tRNA and its involvement in protein synthesis have been the subject of many investigations. Transfer RNAs are small, compact molecules. In the crystallized state, yeast $tRNA^{phe}$ has a

length of 77 Å and an average thickness of 20 Å (Kim et al., 1973). A Stokes's radius of 34 Å was derived for $tRNA^{phe}$ from its translational diffusion coefficient in water (Peters, 1986). A molecule having these dimensions will not readily diffuse through the nuclear pores. Injection of the cloned $tRNA^{tyr}$ yeast gene into the nuclei of oocytes produces a precursor transcript with a length of 108 nucleotides (Melton et al., 1980). After processing, mature tRNA (78 nucleotides long) is then exported into the cytoplasm. De Robertis et al. (1982) injected labelled tRNA into the cytoplasm of oocytes and showed it to be excluded from the nucleus, suggesting that tRNA efflux is vectorial (unidirectional).

Zasloff (1983) studied tRNA transport in amphibian oocytes. This is a convenient system since oocytes can transcribe genes that have been injected into the nucleus. In addition, analysis is facilitated by the fact that nucleus and cytoplasm can be readily separated. Two genes were used in this study; the first was normal human $tRNA^{met}$ and the second was a variant human $tRNA^{met}$ with a G-to-U substitution at position 57 in the highly conserved loop IV region. Nuclei were injected with either the genes encoding for the two tRNAs or prelabelled tRNAs, and the cells were fractionated at various times thereafter. It was found by electrophoretic analysis of both the nucleus and cytoplasm that 80% of the normal tRNA entered the cytoplasm within 5 min; in contrast, 90% of the variant tRNA remained intranuclear after 15 min. Precise kinetic analysis, performed by liquid scintillometry, showed the rate of transport of the variant tRNA to be 20-fold slower than normal tRNA. In addition, normal tRNA transport is saturable and displays

temperature dependence. Furthermore, $tRNA^{phe}$ can act as an effective competitor of $tRNA^{met}$ transport. These results strongly suggest that tRNA transport into the cytoplasm involves a carrier-mediated process rather than simple diffusion.

In an attempt to define the critical region of the tRNA molecule required for transport, Tobian et al. (1985) used site-directed mutagenesis to obtain 30 different mutants with single nucleotide substitutions. Many of the mutations perturbed the rate of transport. The most deleterious effects were in the highly conserved T stem-loop regions; however, many of these tRNAs had impaired processing. It is suggested from this study that transport, in addition to processing, is dependent on maintenance of the secondary structure.

The nuclear pores can serve as diffusion pathways for exogenous tracers and as a transport pathway for some karyophilic proteins. As mentioned above, the diffusion of tRNA out of the nucleus seems unlikely due to i) its size and ii) the involvement of a carrier-mediated process. Zasloff (1983) has suggested that the pores serve as the pathway for exchange and that peripheral ribosome-like particles on the cytoplasmic surface of the nuclear envelope provide the motor mechanism required for transport.

Small nuclear RNAs

The snRNAs comprise 0.1-1.0% of the total cellular RNA. They are small (100-250 nucleotides), stable, uridylic-acid rich RNAs that are localized in the nucleus and are transiently found in the cytoplasm. At least eight snRNAs, U1-U8, have been identified, and all are associated

with a core of five common proteins. Functional roles that have been identified for a few of the U-snRNAs include polyadenylation, intranuclear transport, and splicing of hnRNA.

To investigate intracellular RNA transport within intact cells, De Robertis et al. (1982) injected radiolabelled RNA from HeLa cells into the cytoplasm of Xenopus oocytes. The results demonstrate that snRNAs can migrate from the cytoplasm into the nucleus and concentrate there 30- to 60-fold. It was also established that HeLa snRNAs complex with oocyte proteins in the cytoplasm before their migration into the nucleus. Zeller et al. (1983) analyzed the distribution of snRNAs and their associated binding proteins during oogenesis and early development. Mature Xenopus oocytes and embryos prior to gastrulation are known to contain an excess of snRNA binding proteins (De Robertis et al., 1982) and less U1 and U2 snRNAs than previtellogenic oocytes. In embryos, the midblastula transition (Newport and Kirschner, 1982) marks the onset of a burst in snRNA synthesis and the migration of snRNA binding proteins into the nucleus. Injection of labelled snRNA into the cytoplasm of mature oocytes can prematurely displace the cytoplasmic binding proteins into the nucleus, suggesting that movement of the proteins requires the formation of the snRNPs. To determine whether nuclear accumulation occurs only when snRNA and its binding proteins are complexed together, Mattaj and De Robertis (1985) used site-directed mutagenesis to delete the protein binding site on U2 snRNA. As a result of the snRNA's inability to bind its cytoplasmic proteins, it becomes unable to enter the nucleus. It is suggested that the formation of the snRNP within the cytoplasm might unmask a cryptic targeting signal which

then allows for the accumulation within the nucleus. Whether the signal is in the protein, the RNA, or possibly a combination of both, has yet to be determined.

Ribosomal RNA

The ribosomal DNA transcript, located in the nucleoli, contains the 18S, 5.8S, and 28S genes. After transcription, the rRNA is complexed with proteins to form a 80S ribosomal ribonucleoprotein particle, which is processed to the mature 40S and 60S particles prior to cytoplasmic transport. The 5S RNA is transcribed, processed, and transported separately. These rRNPs are the major components involved in ribosome biogenesis.

Previtellogenic oocytes (stages I and II; Dumont, 1972) synthesize and store 5S RNA which comprises 30-40% of the total cellular RNA. TFIIIA is a transcription factor with a molecular weight of 39 kd that binds to 5S genes. In addition, TFIIIA also binds to cytoplasmic 5S RNA to form a 7S ribonucleoprotein particle. By employing immunocytochemical procedures on sections of Xenopus ovaries, Mattaj et al. (1983) demonstrated that TFIIIA is located predominantly in the cytoplasm. In previtellogenic oocytes, TFIIIA is associated with the 5S RNA (Picard and Wegnez, 1979); thus, the immunocytochemical staining patterns are actually localizing 7S RNPs. Pelham and Brown (1980) studied the in vitro transcription of the 5S RNA gene and showed that transcription could be inhibited when 5S RNA, in excess of the 5S gene, competed for TFIIIA binding. They suggested from these results that TFIIIA might be involved in a feed-back mechanism to control 5S gene expression. It is

important to mention that TFIIIA alone can enter the nucleus to bind to the 5S gene; however, when bound to 5S RNA, it remains in the cytoplasm. Free 5S RNA also is capable of entering the nucleus and it accumulates in the nucleoli (De Robertis et al., 1982). The 7S RNP is able to diffuse throughout the cytoplasm of oocytes (Ford, 1971) but is excluded from the nucleus. The mechanism of exclusion is not yet known; however, it is possible that the formation of the 7S RNP in the cytoplasm masks a transport signal, either on the RNA or the protein that is required for nuclear entry.

Wunderlich and co-workers have studied 18S and 28S rRNA transport from isolated Tetrahymena macronuclei. They reported that rRNP efflux is ATP dependent and the amount of RNP transport is sensitive to a shift in temperature (Giese and Wunderlich, 1983). It was also found that rRNA is integrated into the nuclear matrix which might control the transport process since removal of the nuclear envelope had no effect on the temperature dependence of rRNP export (Wunderlich et al., 1983). Although 28S and 18S RNA leave the nucleus complexed with ribosomal proteins, it is not clear whether the RNA or protein contains the efflux signals.

The role of the nuclear envelope in regulating rRNA translocation was investigated in vivo (Feldherr, 1980) by disrupting the nuclear envelope with glass needles, and in vitro (Stuart et al., 1977) by using membrane-denuded nuclei. The efflux of rRNA from the nucleus to the cytoplasm was not affected by either experimental procedure suggesting that rRNA is most likely bound to nondiffusible nuclear elements.

Overall, tRNA, 5S RNA, and rRNA are generally believed to translocate into the cytoplasm through the nuclear pores. However, much of the evidence is circumstantial and there are no definitive data to indicate that the pores serve as the major pathways for the efflux of these different classes of RNA.

Messenger RNA

Heterogeneous nuclear RNA is processed and undergoes extensive posttranscriptional modifications before mature mRNA can be transported into the cytoplasm. Unspliced mRNA is retained in the nucleus and only mature mRNA appears in the efflux supernatant from isolated nuclei *in vitro* or in the cytoplasm of intact cells, suggesting that processing is one of the many requirements necessary for export. The movement of mature mRNA, from its site(s) of transcription and processing, to the nuclear envelope is the first step involved in export (see above). Agutter (1985a) has suggested that mRNA might not be freely diffusible throughout the cells. To determine whether RNA is preferentially associated with the nuclear matrix, nuclei were treated with nonionic detergents, high salt, EDTA, and urea. It was shown that greater than 90% of the nuclear RNA was tightly associated with the insoluble nuclear matrix (Berezney, 1980; van Eekelen and van Venrooij, 1981). Release of the mature ovalbumin mRNA but not the immature mRNA from the nuclear matrix occurs in the presence of ATP and does not require hydrolysis of the phosphodiester bond (Schroder et al., 1987). This selective detachment from the matrix might be another level of control in regulating the transport of mRNA. Overall, the migration of mature mRNA

to the envelope is likely to involve the processing of the precursor, posttranscriptional modification, and release from the nuclear matrix. Once at the inner surface of the nuclear envelope, the second step is the translocation across the envelope, presumably through the pores.

Translocation of mRNA across the nuclear envelope appears to involve a series of reactions requiring nucleoside triphosphatase (NTPase), protein kinase, protein phosphohydrolase, and poly(A) binding sites. Using isolated nuclei, Agutter et al. (1976) first reported a correlation between RNA efflux and the presence of NTPase activity which was localized in nuclear envelope fractions. Further studies on the energy requirement of poly(A)$^+$mRNA translocation were performed by Clawson et al. (1978). These investigations determined that both ATP and GTP could serve as energy sources.

Clawson et al. (1984) used photoaffinity labelling on rat liver nuclear envelopes and localized the NTPase activity to a 46 kd protein. Schroder et al. (1986a) subsequently purified and characterized a 40 kd NTPase from rat liver nuclear envelopes which is thought to be the same polypeptide identified by Clawson. In addition to the nuclear envelope, NTPase activity was also found to be present in the matrix (Clawson et al., 1984; Maul and Baglia, 1983). There are several experiments that correlate the energy-dependent transport of poly(A)$^+$mRNA with the NTPase activity. First, an increase in the rate of RNA efflux by thioacetamide treatment (Clawson et al., 1980), insulin administration (Purrello et al., 1982), or tryptophan feeding (Murty et al., 1980), causes a parallel increase in NTPase activity. Second, in a transport assay using isolated nuclei, NTPase activity and mRNA efflux have similar

kinetic properties, substrate specificities, and sensitivities to inhibitors (Agutter et al., 1976; Clawson et al., 1978, 1980). One additional line of evidence that mRNA and NTPase are interrelated is the inhibition of mRNA efflux by antibodies against pore complex-lamina components that affect NTPase activity (Schroder et al.; unpublished results).

The effects of poly(A) on NTPase activity were observed initially by Agutter et al. (1977) on isolated, intact nuclear envelopes. They showed that addition of poly(A) or poly(G) to the media could enhance NTPase activity. Furthermore, Bernd et al. (1982) demonstrated the NTPase activity to be markedly stimulated by either poly(A)$^+$mRNA or a homopolymer of poly(A) at least 15-20 bases long. Additional evidence comes from experiments by Riedel and Fasold (1987). They prepared resealed nuclear-envelope vesicles that retain NTPase activity and found that both poly(A) and mRNA can stimulate the NTPase activity associated with these vesicles. It is suggested from these data that poly(A) can modulate NTPase activity and possibly regulate the translocation of mRNA.

It has been demonstrated that protein kinases and phosphohydrolases are present in the nuclear envelope (Lam and Kasper, 1979; Smith and Wells, 1983; Steer et al., 1979a, 1979b). McDonald and Agutter (1980) have studied the effects of polyribonucleotide binding on the phosphorylation and dephosphorylation of nuclear envelope proteins and found that the same polyribonucleotides that stimulate NTPase, inhibit protein kinase activity and stimulate one of the phosphohydrolases. They also found the nuclear envelope to contain a population of poly(A)

binding sites that increase in affinity by a kinase-dependent phosphorylation reaction. The actual role these components play in the in vivo translocation of mRNA across the envelope has not yet been clearly defined; however, poly(A)$^+$mRNA efflux models have been proposed by Agutter (1985a) and Schroder et al. (1988).

The model proposed by Agutter (1985a) integrates the components involved in RNA translocation: NTPase, poly(A) binding sites, protein kinases and phosphohydrolase, to explain nucleocytoplasmic transport of poly(A)$^+$mRNA. The following scheme is based on Agutter's model (1985a). The NTPase exists in a complex with the poly(A) binding site. A protein kinase uses MgATP as a substrate to phosphorylate the poly(A) binding site. Binding of nuclear poly(A)$^+$mRNA to the [phosphorylated binding site-NTPase] complex stimulates dephosphorylation by the phosphohydrolase. MgATP is also a substrate for NTPase, the binding of MgATP to the NTPase of the [poly(A)$^+$mRNA-poly(A) binding site-NTPase] complex causes the displacement of poly(A)$^+$mRNA, thus facilitating the translocation of mRNA. The NTPase then hydrolyses ATP and the [NTPase-poly(A) binding site] complex is available again to start another cycle.

At least two cytosolic proteins, 34 kd and 58 kd, with an affinity for poly(A) can stimulate mRNA efflux in vitro (Moffett and Webb, 1981, 1983; Schroder et al., 1986b). The 34 kd protein, in the presence of poly(A), stimulates the NTPase activity to a greater extent than poly(A) by itself. In the absence of poly(A), p34 has no effect on kinase or phosphohydrolase activity. The 34 kd protein is thought to enhance NTPase activity by increasing the affinity of poly(A) to the poly(A) binding site in the phosphorylated nuclear envelope. Schroder et al.

(1988) suggest that the 58 kd protein inhibits kinase activity by promoting poly(A) binding to unphosphorylated envelopes, which prevents the down-regulation of NTPase activity by the kinase.

The third step in the overall RNA transport is the involvement of the cytoplasm. Although it is established that much of the mRNA is bound to the cytoskeleton (Jeffery, 1982), it is not clear how the RNA finds its final destination. The proteins associated with poly(A)$^+$mRNA in the cytoplasm are different from those that are bound to poly(A) within the nucleus (Baer and Kornberg, 1983; Sachs and Kornberg, 1985; Setyono and Greenberg, 1981). It is not known if these proteins are involved in the transport of RNAs within the cytoplasm or serve as anchors to cytoskeletal elements. In this regard, it is necessary to further understand the functional role of the cytoplasmic proteins associated with RNAs.

Ribonucleoproteins (RNPs) have been visualized exiting the nucleus through the nuclear pores by electron microscopic cytochemical analysis (Stevens and Swift, 1966). In a more recent study, Skoglund et al. (1983) studied the formation and transport of an hnRNP particle, a transcription product from the Balbiani ring genes in Chironomus. Their results, by using the Miller spread technique, describe the growth of a 100 Å wide filament into a large 400-500 Å granule. Prior to export, these mature RNP particles undergo conformational changes that result in the formation of rod-shaped structures with an average length of 1350 Å and diameters of 250-300 Å that pass through the centers of the nuclear pores. Even though these RNPs undergo a shape change, they are still

too large to pass through the pores by passive diffusion, suggesting that some facilitated mechanism is a requirement for export.

Statement of Research Topic

It has been demonstrated that there are selective mechanisms which facilitate the passage of certain endogenous macromolecules through the nuclear pores. The mechanism and selectivity of the pore by which transport occurs is not known; for example, does translocation proceed through the pores by a gated mechanism, or does transport occur through a fixed channel? How does the nuclear targeting signal interact with the pore region to initiate translocation? Do different nuclear transport signals use the same pore for uptake? What effects do variations in the number and sequence of targeting signals have on nuclear uptake? In relation to RNA transport, what is the precise location for the translocation of different classes of RNA to the cytoplasm? Are there specific functional classes of pores that are involved in RNA transport? or do all pores have the capability to translocate both RNAs and proteins?

To better understand the role of the nuclear envelope in regulating nucleocytoplasmic exchange, experiments were designed to elucidate characteristics of the macromolecular transport process. The data obtained will focus on the translocation of RNAs. More specifically, analysis will include the proportion of functional pores, the precise location and size of the transport channels, and the multifunctional capability of individual pores (i.e., both protein uptake and RNA efflux). In addition, data will be obtained for the effect of different

numbers of synthetic nuclear targeting signals on protein uptake and the functional size of the transport channel. It will also be determined whether individual pores have the capability to transport proteins containing different nuclear targeting sequences.

Many of the previous nucleocytoplasmic exchange experiments were based on quantitative and qualitative studies performed with fluorescein- or radio-labelled molecules; however, there are certain characteristics of the exchange sites that cannot be studied using these type of tracer molecules. In this study, colloidal gold particles will be used to identify and characterize the sites for macromolecular exchange.

Colloidal gold tracers have some unique properties that make them ideal for this type of experimentation. First, gold particles with different diameters can be easily prepared. Second, the surface properties of the particles can be modified by the adsorption of different molecules and the particles will then acquire the characteristics of their coating agents. Third, tracer particles can be localized precisely to specific cell structures by electron microscopy.

The following study will focus primarily on nucleocytoplasmic transport of macromolecules, especially with regard to the characteristics of the exchange sites located within the nuclear pores. The experiments are performed on amphibian oocytes and involve the microinjection of either RNA- or protein-coated gold particles into the nuclear or cytoplasmic compartment of the cells. The subsequent intracellular distribution of the tracer particles and their relation to the nuclear pores will be determined by electron microscopy.

CHAPTER II
TRANSLOCATION OF RNA-COATED GOLD PARTICLES THROUGH THE NUCLEAR PORES OF OOCYTES

Introduction

The nuclear envelope in eukaryotic cells is the site of continual macromolecular exchange between the nucleoplasm and the cytoplasm. Such exchanges can occur either by passive diffusion or mediated transport through the nuclear pores (Dingwall and Laskey, 1986). Microinjection of various sized exogenous molecules has been used in previous studies to determine the dimensions of the diffusion channels within the pores. The channels were estimated to be 90 Å in diameter in amphibian oocytes (Paine et al., 1975), and are available for diffusion both into and out of the nucleus (Paine, 1975). Evidence for protein transport across the envelope was initially obtained for RN1 (Feldherr et al., 1983) and nucleoplasmin (Dingwall et al., 1982), both of which are major karyophilic proteins found in amphibian oocytes. The specific sites of transport for nucleoplasmin have been identified by microinjecting nucleoplasmin-coated gold particles into the cytoplasm of the oocytes (Feldherr et al., 1984). It was found, by electron microscopy, that transport of the colloidal particles occurred through channels, at least 200 Å in diameter, located in the centers of the nuclear pores. Thus, the regions of the pores available for transport are considerably larger than those available for diffusion.

The exit of mRNA from isolated nuclei has been studied in a number of laboratories (reviewed by Clawson et al., 1985). These investigations have shown that efflux is a temperature-dependent, energy-requiring process, indicating that some form of transport is involved (Agutter, 1985a; Clawson et al., 1978). In support of this view, isolated nuclear envelope preparations were found to contain nucleoside triphosphatase activity which is affected by the same factors (ionic composition, pH, etc.) that regulate RNA efflux (Agutter, 1985a; Clawson et al., 1980; 1984). It has also been suggested, on the basis of results obtained in several laboratories, that the poly(A) tail of mRNA is involved in transport across the envelope (Agutter, 1985b; Bernd et al., 1982).

The efflux of tRNA from the nucleus has been investigated by Zasloff (1983) using in vivo procedures. He obtained evidence for a saturable, carrier-mediated transport mechanism for $tRNA^{met}$ in the amphibian oocyte. De Robertis et al. (1982) have demonstrated that microinjected 5S RNA can migrate either into or out of the nucleus in Xenopus oocytes; however, the mechanism of exchange was not determined.

It is generally believed that RNA transport occurs through the nuclear pores. Indirect evidence supporting this view has been reviewed by Franke and Scheer (1974); direct evidence was obtained by Stevens and Swift (1966). These latter investigators observed that 400 Å RNP granules synthesized at the Balbiani rings in Chironomus salivary gland cells exit through the pores. As the particles enter the pores they are transformed into rod-like structures with diameters of about 200-250 Å.

There is evidence that RNA is not freely diffusible within the nucleus and cytoplasm but is associated with structural elements of the nuclear matrix and cytoskeleton (Agutter, 1985a; Feldherr, 1980; Fey et al., 1986; van Eekelen and van Venrooij, 1981). Based partly on these findings, Agutter (1985a) postulated that the efflux of RNA occurs in three stages by a solid-phase mechanism; first, transport to the nuclear envelope along the nuclear matrix, second, translocation through the pores, and third, transport along the cytoskeletal matrix.

The focus of the present investigation is on the second step of the efflux process, i.e., translocation across the envelope. The main objectives are; first, to localize and characterize the specific regions of the nuclear pores involved in the translocation of different classes of RNA-coated gold to the cytoplasm and, second, to determine whether individual pores can function in both RNA efflux and protein uptake, or whether separate classes of nuclear pores exist. In order to obtain the resolution necessary to examine individual exchange sites, coated colloidal gold particles are used as tracers. The particles are microinjected into oocytes, and subsequently localized by electron microscopy.

The results are summarized as follows: first, gold particles coated with tRNA (met or phe), 5S RNA, or poly(A) are all translocated into the cytoplasm through central channels, at least 230 Å in diameter, located within the nuclear pores. Particles stabilized with nonphysiological polynucleotides are also translocated; however, the number and distribution of the particles associated with the pores varied for different coating agents. Second, approximately 97% of the

pores in Xenopus oocytes can function in the translocation of RNA-coated gold. Third, the accumulation of particles in the pores is a saturable process. Fourth, individual pores can function in both RNA efflux and protein uptake. Control experiments ruled out the possibilities of fixation artifacts and non-specific exchange processes.

Materials and Methods

Xenopus laevis were purchased from Xenopus 1 (Ann Arbor, Michigan) and maintained as reported previously (Feldherr, 1975).

Nucleoplasmin isolation

Nucleoplasmin was isolated from a starting volume of 30 ml of Xenopus ovaries. The isolation procedure was similar to that described by Dingwall et al. (1982), with the exception that an anti-nucleoplasmin IgG affinity column was substituted for the DEAE-cellulose and phenyl sepharose columns. Polyclonal antibodies against nucleoplasmin were generated in rabbits and affinity purified. A cleared cell homogenate, obtained from the lysed oocytes (Feldherr et al., 1984), was passed over an anti-nucleoplasmin affinity column, and the column was washed free of nonbound proteins. Nucleoplasmin was eluted in 1 ml fractions with 50 mM glycine-HCl buffer (pH 2.5) into microcentrifuge tubes containing sufficient 1 M tris (pH 9.0) to increase the pH to 7.5. The protein was monitored at 280 nm, and the fractions containing nucleoplasmin were pooled and treated with ammonium sulfate (55% saturated) overnight in the cold. The soluble $(NH_4)_2SO_4$ fraction was then dialyzed against a solution containing

0.05 M Tris and 0.05 M NaCl (pH 7.2), after which the nucleoplasmin was precipitated in 80% alcohol and lyophilized as described previously (Feldherr et al., 1984). Gel analysis was performed, as described by Laemmeli (1970), to determine the purity of the preparation.

Gold preparation and stabilization

All glassware and solutions used in experiments involving RNA were treated with 0.01% (vol/vol) diethyl pyrocarbonate and then autoclaved. Colloidal gold particles were prepared by reducing chloroauric acid with either trisodium citrate (Frens, 1973) or a saturated solution of white phosphorus in ether (Feldherr, 1965). In this study, the trisodium citrate method gave a particle distribution of 120-220 Å in diameter, whereas the phosphorus ether preparations ranged from either 20-50 Å or 20-160 Å, depending on the initial concentration of gold chloride used.

The gold sols were stabilized with tRNA (met or phe), 5S RNA, poly(A) (3500 bases), poly(I) (500 bases), poly(dA) (500 bases), nucleoplasmin, polyvinylpyrrolidone (PVP; 40 kd), polyglutamic acid, ovalbumin, or bovine serum albumin (BSA). The RNAs were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) or Sigma Chemical Company (St. Louis, MO). The purity of the RNAs was tested by running the preparations on 10% polyacrylamide gels containing 8.3 M ultrapure urea; ethidium bromide was used as a stain. Polyglutamic acid, BSA, ovalbumin and PVP were purchased from Sigma Chemical Company.

In each instance, the minimum amount of coating agent required to stabilize the particles, that is, prevent precipitation in 1% NaCl, was determined as described earlier (Feldherr, 1984). Before stabilization,

tRNA, poly(A), poly(I), and poly(dA) were dissolved in 10 mM KCl, 7.2 mM K_2HPO_4 and 4.8 mM KH_2PO_4 (pH 7.0). 5S RNA was rehydrated in 2 ml of sterile ion-free water resulting in a salt concentration of 1 mM Tris-HCl, 10 mM NaCl and 0.1 mM $MgCl_2$ (pH 7.5). Nucleoplasmin, polyglutamic acid, PVP, BSA, and ovalbumin were solubilized in a low ionic strength buffer containing 7.2 mM K_2HPO_4 and 4.8 mM kH_2PO_4 (pH 7.0). Concentrations of coating agents and volumes used to stabilize the gold preparations are given in Table I.

After stabilization, the 20-160 Å preparations were centrifuged at 6,000g (at the bottom of the tube) at 4°C for 10 minutes to remove any large aggregates of gold. This step was not necessary for the 20-50 Å and 120-220 Å fractions. Five to 7 ml of stabilized colloid were then concentrated to 70-100 µl in Minicon concentrators (Amicon Corp., Danvers, MA). Finally, the samples were dialyzed against intracellular medium consisting of 102 mM KCl, 11.1 mM NaCl, 7.2 mM K_2HPO_4, and 4.8 mM KH_2PO_4 (pH 7.0) for 3 h at 4°C.

Injection

Frogs were anesthetized on ice for one hour and the ovaries removed. Late stage 5 and stage 6 oocytes (Dumont, 1972) were manually defolliculated with watchmakers forceps and maintained in Ringer's solution (Diberardino et al., 1977) at 22°C. The defolliculated cells were centrifuged at approximately 650g for 8-10 minutes as described previously (Feldherr, 1980; Kressmann and Bernstiel, 1980). During centrifugation, the nucleus migrates to a position just underneath the plasma membrane at the animal pole and its outline can be visualized due

Table I - Amounts of Coating Agent Required to Stabilize Gold Sols

Coating agent	mg of coating agent/ml of stabilizing solution	Size range of colloidal particles stabilized (Å)	µl of solution needed to stabilize 1 ml of colloid
$tRNA^{met}$	0.2	20-160	200
		20- 50	50
$tRNA^{phe}$	0.5	20-160	300
5S RNA	0.4	20-160	400
poly(A)	0.5	20-160	70
		120-220	70
poly(dA)	0.25	20-160	350
poly(I)	0.5	20-160	400
Nucleoplasmin	0.1	120-220	60
PVP	0.1	20- 50	40
BSA	1.0	20- 50	60
Ovalbumin	1.0	20-160	250
Polyglutamic acid	10	20- 50	200

*These are average values, intended to serve as a guide. The exact amounts of coating agents required for stabilization should be determined for each individual gold preparation.

to the displacement of pigment granules in the cortex. Nuclear injections could then be accomplished. Calcium-free Ringer's was used as an extracellular medium during injection to prevent possible precipitations of the colloid as the micropipettes were introduced into the cells. Immediately after injection the cells were returned to complete Ringer's solution until subsequent fixation. The total exposure to calcium-free Ringer's was less than 30 minutes. The tip diameters of the micropipettes were 10-15 μm.

Electron microscopy and analysis

The cells were fixed using a procedure similar to that described by Kalt and Tandler (1971). The oocytes were fixed initially overnight at 4°C in 100 mM HEPES containing 3% glutaraldehyde (vol/vol), 2% paraformaldehyde (wt/vol), 2.5% DMSO (vol/vol) and 1 mM $CaCl_2$ (pH 7.2). The nuclei were then dissected out with their surrounding cytoplasm, post-fixed in 2% OsO_4 (wt/vol) for one hour, and stained with 0.5% p-phenylenediamine (wt/vol) in 70% acetone for 30 minutes. Finally, the samples were dehydrated in a graded series of acetone and embedded in Spurr's medium. Thin sections were cut on a Reichert microtome and analyzed with a JEOL 100S electron microscope (JEOL USA, Cranford, NJ).

In the RNA efflux studies it was presumed that gold particles located within the pores were in the process of translocation, and that particles present in the cytoplasm just adjacent to the pores had completed the translocation process. These areas will be referred to as regions 1 and 2, respectively (see Fig. 1). Pores that contained one or more particles in either or both of these regions were considered to be

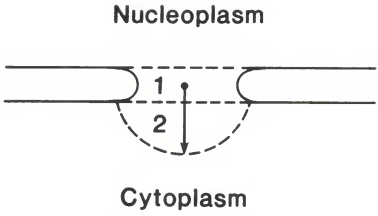


Figure 1. A schematic representation of the regions used for electron microscopic analysis of the particle distribution within the pores. It is assumed that the particles in region 1 are undergoing translocation and that particles in region 2 have completed the process. The arrow which defines the outer limits of region 2 represents a distance of approximately 500 Å.

actively involved in efflux. To determine the functional diameter of the transport channels, the size distribution of the particles in regions 1 and 2 were obtained and compared with the size of the injected particles (i.e., particles within the nucleus). To standardize the results, pores were analyzed in regions where the gold concentration in the adjacent nucleoplasm was about 100 particles/0.36μm^2.

Negative staining procedures were used to estimate the overall diameters of the particles, that is, the gold plus the adsorbed coat material.

Results

Purity of stabilizing agents

Approximately 600 µg of nucleoplasmin was isolated from 30 ml of ovary using affinity chromatography. SDS-polyacrylamide gels of the isolated protein showed major bands with apparent molecular weights of 165 kd and 145 kd, and a minor band with a molecular weight of approximately 33 kd. The gel pattern (not shown) was identical to that obtained for nucleoplasmin isolated using DEAE and phenyl sepharose columns (Feldherr et al., 1984).

On a 10% polyacrylamide gel containing urea, tRNA ran as a single distinct band corresponding to approximately 70 nucleotides, whereas the pattern obtained for 5S RNA contained one major band corresponding to approximately 130 nucleotides and minor bands of smaller sizes, probably representing breakdown products. Gel scans of the tRNA and 5S RNA showed the major band in each sample to be >80% of the total RNA.

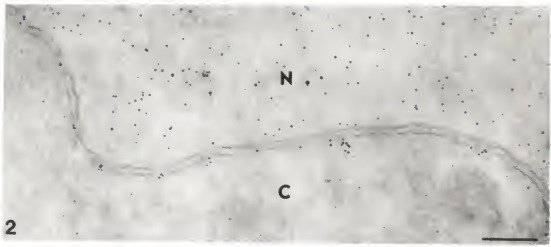
Nuclear injection of tRNA-, 5S RNA- and poly(A)-coated gold

It was determined initially that varying the amount of the injectate from 8-20 nl (the RNA content ranged from 30-100 ng) had no effect on the results. Routinely, 8 nl of colloid were injected. This volume contained a sufficient number of particles for electron microscopic analysis and minimized possible damage to the nucleus. The oocytes were fixed 15 min, 1 h, or 6 h after injection. Eighteen to 20 cells were examined for each type of RNA at each time interval.

The results were essentially the same for 20-160 Å particles coated with tRNA (met or phe), 5S RNA, or poly(A). At all time intervals, particles were distributed randomly throughout the nucleoplasm, except for aggregates of gold that were occasionally observed at the surface of the nucleoli. After 15 min and 1 h, particles were associated with almost all of the nuclear pores. Representative results for tRNA and 5S RNA are shown in Figs. 2 and 3, respectively. Based on the assumption that the presence of gold particles within the pores or in the adjacent cytoplasm (regions 1 and 2 in Fig. 1) is indicative of nucleocytoplasmic exchange, it was concluded that over 97% of the pores were involved in translocation 15 min and 1 h after injection (see Table II). There was an obvious decrease in the percentage of pores that contained particles in the 6 h experiments, but these results were not quantitated. In all instances, particles were observed in the cytoplasm beyond the immediate vicinity of the pores (i.e., beyond region 2). However, even after 6 h the cytoplasmic to nuclear concentration ratio was only 1:14.

Figure 2. tRNA-gold, nuclear injection - 15 min experiment. Gold particles (20-160 Å) are observed evenly distributed throughout the nucleus (N). Particles are seen within the centers of the majority of the pores and also in the adjacent cytoplasm (C). Bar, 0.2 μm.

Figure 3. 5S RNA-gold, nuclear injection - 60 min experiment. The tracer particles (20-160 Å) show a similar distribution as described in Figure 2. Particles are present within the pores and the adjacent cytoplasm (C). N, nucleus. Bar, 0.2 μm.


N
C
2

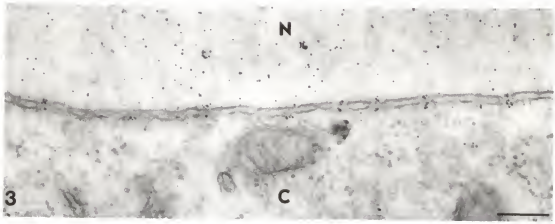

N
C
3

Table II - Translocation of Gold Particles as a Function of the Coating Agent

Experiment (1 h)	No. of Particles Translocated*			Particles/Pore	% of Pores Active in Translocation*
	Region 1	Region 2	Total		
tRNA-gold	437	414	851	4.3	97%
5S RNA-gold	458	425	883	4.4	98%
Poly(A)-gold	309	479	788	3.9	98%
Poly(dA)-gold	334	144	478	2.4	98%
Poly(I)-gold	397	543	940	4.7	96%
PVP-gold**	3	5	8	0.04	4%
BSA-gold**	5	3	8	0.04	4%
Ovalbumin-gold	2	9	11	0.05	6%

* Data based on the analysis of 200 pores all within equivalent concentrations of gold particles.

**The size of the gold particles in these experiments ranged from 20-50 Å. In all other experiments the particle sizes ranged from 20-160 Å.

The size distributions of tRNA- and 5S RNA-coated particles present within the nucleoplasm and also associated with the nuclear pores (regions 1 and 2) are given in Table III. Since the thickness of the RNA coat, in both cases, was estimated to be 15-20 Å, the results indicate that particles with an overall diameter of at least 170 Å can pass through the centers of the pores. Since larger particles could not be stabilized with tRNA or 5S RNA, it was not possible to determine an upper size limit for translocation using these coating agents. In contrast, 120-220 Å particles could be stabilized with poly(A). The results obtained wtih poly(A)-gold are illustrated in Fig. 4. Despite the fact that relatively few particles were injected into the nuclei, gold was found in over 70% of the pores after 15 min and 1 h. The size distribution of poly(A)-coated particles associated with the pores is shown in Table III. It is apparent from the data, which is based on the examination of 12 cells, that particles at least 230 Å in diameter (including the coat) are translocated through the pores.

Nuclear injection of poly(I)- and poly(dA)-coated gold

In addition to the studies performed with tRNA, 5S RNA and poly(A), nuclei were also injected with poly(I)- or poly(dA)-coated particles. The volumes injected, and the procedure used for analysis of these nonphysiological tracers, were the same as described above. In these experiments, the oocytes were fixed 1 h after injection, and 5 cells were examined in each group. The results are shown in Table II. Particles coated with either poly(I) or poly(dA) were translocated through the centers of the pores; however, differences were observed in

Table III - Size Distribution of Gold Particles Present in the Nuclei and Pores

Experiment (1 h)	Total No. of Particles Measured	% of Particles in Each Size Class*									
		20-40 Å	40-60 Å	60-80 Å	80-100 Å	100-120 Å	120-140 Å	140-160 Å	160-180 Å	180-200 Å	>200 Å
$tRNA^{met}$											
Nuclei**	486	0.3	27.9	51.5	12.8	4.2	1.5	1.5	--	--	--
Pores	462	0.4	22.1	52.6	15.4	6.1	1.3	2.1	--	--	--
5S RNA											
Nuclei	1110	3.2	35.8	48.4	10.0	1.8	0.45	0.36	--	--	--
Pores	773	2.6	37.0	48.4	10.3	1.3	0.25	0.15	--	--	--
Poly (A)											
Nuclei	906	--	--	--	--	--	6.6	35.5	43.8	9.8	4.2
Pores	593	--	--	--	--	--	5.2	37.3	42.0	13.6	1.9

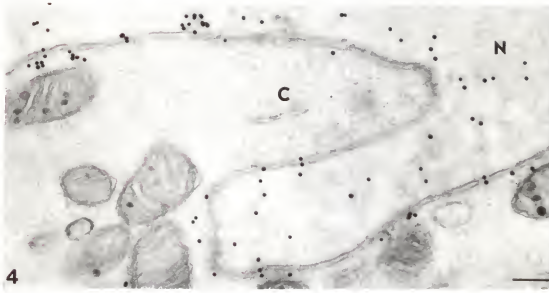
* Particle dimensions do not include the thickness of the coat. Negative staining indicated that the coat thickness adds 30-40 Å to the overall diameters of the particles.

** Particles present within the nuclei.

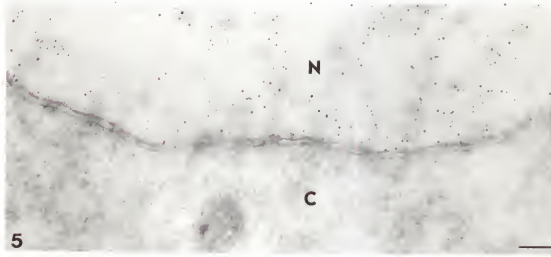
*** Total number of particles present within the pores and in the cytoplasm immediately adjacent to the pores.

Figure 4. Poly(A)-gold, nuclear injection - 60 min experiment. Large gold particles (120-220 Å) are observed extending through the nulcear pores and are present in the adjacent cytoplasm (C). N, nucleus. Bar, 0.2µm.

Figure 5. PVP-gold, nuclear injection - 60 min experiment. Gold particles are retained within the nucleoplasm and are rarely seen associated with the envelope or within the nuclear pores. C, cytoplasm. N, nucleus. Bar, 0.2 µm.



4



5

the numbers and distribution of the particles associated with these structures. Compared with poly(dA)-coated gold, almost twice as many poly(I)-coated particles were present in the pore areas and, in addition, a higher proportion of these particles were located in region 2. These results suggest that some polynucleotides are translocated more efficiently than others.

Nuclear injection of BSA-, ovalbumin-, polyglutamic acid-, and PVP-coated gold

To determine if the translocation of RNA-gold is a selective process, nuclei were injected with gold fractions that had been stabilized with the exogenous molecules PVP, BSA, or ovalbumin. The volumes injected, and the experimental times (15 min, 1 h and 6 h), were the same as those used in the RNA studies. The data are based on the examination of 6-9 cells per time interval for each coating agent that was used. The result of a 1 h nuclear PVP-gold injection is illustrated in Fig. 5. Similar distributions were observed when particles stabilized with BSA or ovalbumin were injected. It was found, at all time intervals, that essentially all of the particles coated with exogenous substances were retained within the nuclei and less than 6% of the pores contained gold particles (Table II). It can also be seen in Table II that the total number of control particles translocated per 200 pores was only about 1% of the total number of translocated RNA-coated particles. These results demonstrate that the translocation of RNA-gold was due to the specific properties of the adsorbed coat material.

To determine if the translocation of polynucleotide-coated particles is due simply to a high negative charge density, gold particles were coated with polyglutamic acid, which, like RNA, is a polyanion. Since polyglutamic acid is not a highly effective stabilizing agent, the colloid preparations that were injected were relatively dilute. To compensate for this factor, tRNA-gold preparations having an equivalent particle concentration were injected in parallel experiments. The results, which are based on an analysis of 5 cells, are shown in Table IV. Tracer particles coated with polyglutamic acid were observed in only 8% of the pores, compared with a value of 83% obtained for tRNA-gold. These findings demonstrate that the translocation of the RNA-coated particles is not simply a charge effect.

Controls

Control experiments were performed on 6 oocytes to establish if the results obtained for RNA-gold could be due to a redistribution of the particles during fixation. In this study, the nuclei were injected with tRNA-coated gold and the oocytes fixed within 10 seconds. The particles near the site of injection were rarely observed within the pores or in the adjacent cytoplasm. These data indicate that the presence of RNA-gold within the pores at 15 min and 1 h intervals was not a fixation artifact, but reflected an *in vivo* exchange process.

In order to ascertain whether the presence of excess RNA itself could alter the properties of the pores, nuclei of 6 oocytes were simultaneously injected with PVP-coated gold and 100 ng of soluble tRNA.

Table IV - Translocation of Gold Particles Coated with Polyglutamic Acid

Experiment (1 h)	No. of Particles Translocated			Particles/Pore	% of Pores Active in Translocation
	Region 1	Region 2	Total		
Polyglutamic Acid-gold	21	2	23	0.1	8%
tRNA-gold	175	164	339	1.7	83%

*Data based on the analysis of 200 pores. In both instances the concentration of gold particles (20-50 Å) in the adjacent nucleoplasm was approximately 60 particles/0.36 μm^2.

Fewer than 4% of the 200 pores analyzed were involved in translocation. Thus, the injection of RNA in amounts greater than those normally used in this investigation, have no apparent effect on the physical properties of the pores.

Concentration dependence of RNA translocation

To determine the concentration dependence of the translocation process, the gold distribution in the pores was analyzed in areas in which different concentrations of colloid were present in the adjacent nucleoplasm. These experiments were performed with tRNA-coated gold particles, and the cells were fixed after 1 h. To ensure that an appropriate concentration range was obtained, different dilutions of colloid were injected. The amount of tRNA in the injectate varied from 6-30 ng. The results are shown in Table V, and are based on the examination of 6 oocytes. It can be seen that a maximum number of particles per pore is obtained at a concentration of 90 particles/0.36μm^2. Above this concentration, no differences were observed either in the numbers or distribution of the particles, suggesting that the translocation process is saturable.

Double label experiment

The high percentage of pores involved in RNA translocation suggested that each pore may be a bidirectional channel, capable of both protein and RNA transport. To address this possibility directly, large gold fractions (120-220 Å) coated with nucleoplasmin and small gold fractions (20-50 Å) stabilized with tRNA were used. The tRNA-coated

Table V - Concentration Dependence of tRNA-gold Translocation*

Particle No./0.36 μm^2	No. of Particles Translocated			Particles/Pore	% of Pores Active in Translocation
	Region 1	Region 2	Total		
30	34	84	118	1.2	61%
60	77	94	171	1.7	82%
90	159	197	356	3.6	98%
120	154	205	359	3.6	98%
190	188	174	362	3.6	98%

* Data based on the analysis of 100 pores for each gold particle concentration.

The size of the gold particles ranged from 20-50 Å, and the cells were fixed 1 h after injection.

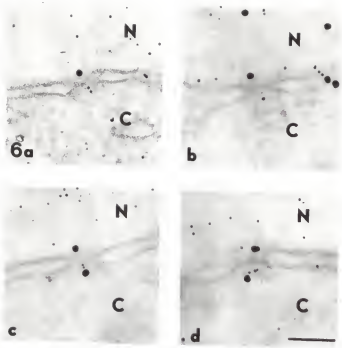
particles were injected into the nucleus and nucleoplasmin-coated gold was injected into the cytoplasm of the same cell. The sequence of the injections varied in different experiments; however, in all instances the interval between the first and second injection was 15 min, and the oocytes were fixed after a total of 45 min. In all, 12 cells were examined. The results, which were the same regardless of the injection sequence, are illustrated in Fig. 6, a-d. Small RNA particles can be observed immediately adjacent to the cytoplasmic surface of the pores and large nucleoplasmin particles are present on the nuclear side of the same pores. These distributions clearly demonstrate that individual pores can function in both protein uptake and RNA efflux.

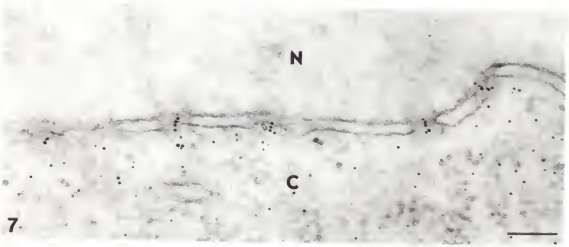
Cytoplasmic injections

The RNA-gold preparations [tRNA, 5S RNA and poly(A)] were injected into the cytoplasm to determine if transport is reversible. Approximately 40 nl of colloid (containing approximately 200 ng of RNA) were introduced adjacent to the nuclear envelope in a total of 20 centrifuged cells. Similar distributions were obtained for all RNAs used. In both 1 h and 6 h experiments, gold was seen within the poresnear the site of injection, but relatively few particles were found in the nucleoplasm. The nuclear to cytoplasmic concentration ratios were approximately 0.01 after 1 h. The 5S RNA-gold results are illustrated in Fig. 7. It should be pointed out that the number of pores that contained gold varied considerably from cell to cell, therefore, no effort was made to quantitate these results.

Figure 6. Double injection experiment - 45 min.
tRNA-gold (20-50 Å) was injected into the nucleus (N), and the adjacent cytoplasm (C) was injected with nucleoplasmin-gold (120-220 Å). The large nucleoplasmin-coated particles can be seen just on the nuclear sides of the pores and small RNA particles are present on the cytoplasmic side. Bar, 0.1 μm.

Figure 7. 5S RNA-gold, cytoplasm injection - 15 min experiment.
Gold particles are present in the cytoplasm (C) and can be seen extending through the nuclear pores. Only a small percentage of particles enter the nucleus (N). Bar, 0.1 μm.


N
C
6a
b
c
d


N
C
7

Discussion

Colloidal gold procedures have been used previously to identify and characterize the regions of the pores that are involved in the transport of karyophilic proteins across the nuclear envelope (Feldherr et al., 1984). It was found that the transport channels are located in the centers of the pores and have functional diameters of at least 200 Å. In this study, the same experimental approach was used to characterize the pathways for RNA efflux and, in addition, to determine whether individual pores have the capability of transporting both RNA and protein.

The presence of RNA-coated gold particles within and along the cytoplasmic surface of the nuclear pores provides direct evidence that these tracers are translocated across the envelope at these sites. Furthermore, almost all of the pores (97% or more) can function in the translocation process. The biological significance of these findings; however, is dependent on demonstrating that the pathways visualized for the colloidal gold tracers are the same as those normally used for the exchange of endogenous RNA. In this regard, we have attempted to demonstrate that the translocation of RNA-gold through the nuclear pores is both a selective and active process, and that the observed gold distributions were not due to technical artifacts.

The fact that gold was not present in the pores of cells fixed 10 seconds after injection shows that accumulation in these structures is not simply a result of the injection procedures per se or a redistribution of the particles during fixation and processing for electron microscopy. Control studies also demonstrated that the

injection of excess RNA does not alter the overall properties of the pores.

Gold particles coated with synthetic polymers (PVP or polyglutamic acid), exogenous proteins (BSA or ovalbumin) and even endogenous karyophilic proteins (nucleoplasmin, Feldherr et al., 1984) are essentially excluded from the pores after nuclear injection, demonstrating that the translocation of RNA-gold is a selective process. The data obtained with poly(I) and poly(dA) indicate that the capacity for translocation is not necessarily restricted to physiologically active RNAs but may be a general property of polynucleotides. The chemical and/or physical characteristics of RNA that facilitate translocation are not known, although a comparison of the results obtained with poly(A) and poly(dA) suggests that the sugar moieties could be involved. The high negative charge density of RNA does not appear to be a major contributing factor since particles coated with polyglutamic acid are largely excluded from the pores.

In view of the results obtained by Zasloff (1983), which showed that the transport of labelled tRNA is markedly reduced by a single substitution of G-to-U at position 57, one might expect a greater degree of specificity for the translocation of polynucleotides. It should be kept in mind, however, that Zasloff measured the overall efflux of RNA whereas our results relate specifically to the translocation step across the envelope. Thus, the decreased rate of transport of the variant is not necessarily due to an effect on translocation through the pores, but could be due to increased binding

within the nucleoplasm or changes in the rate of migration in the cytoplasm.

As discussed in the introduction, there is evidence that macromolecules larger than 90 Å in diameter are unable to diffuse across the nuclear envelope, whereas particles as large as 200 Å in diameter, which contain nuclear targeting signals, can be transported through central channels in the pores. It was found in this study that RNA-coated gold particles as large as 230 Å in diameter (including the coat) readily penetrate the pores. Since this far exceeds the upper limit for diffusion, these results suggest that a transport process is involved. Consistent with this interpretation is the finding that the accumulation of RNA-gold in the pores is a saturable process. Saturation indicates the presence of a carrier-mediated transport process. However, saturation would also occur if the particles simply occupied all of the available space within and adjacent to the pore channels. The latter explanation, which would have little bearing on the mechanism of exchange, is unlikely for two reasons. First, direct electron microscopic examination of region 1 shows that these areas are not fully occupied at saturation levels. Second, particles coated with nucleoplasmin can reach more than twice the concentration in region 2, than tRNA-coated gold (data not shown), demonstrating that the nature of the coat material rather than the total number of tracer particles is the determining factor.

Colloidal tracers, although well suited for localizing and characterizing exchange sites, are not appropriate for detailed kinetic studies, which require numerous time points and involve the analysis of

large samples. For this reason a comprehensive examination of the temperature dependence of translocation was not attempted. However, a cursory study was performed at 4°C and at 21°C, comparing the accumulation and distribution of tRNA-coated gold particles in the pores. After 15 minutes, the average number of particles per pore (based on the analysis of 100 pores) at 21°C and 4°C was 3.8 and 1.5, respectively. Furthermore, at 4°C only 25% of the particles were located in region 2, compared to 50% in cells kept at 21°C. The temperature effects are very likely influenced by two separate processes, binding to receptors and movement through the pores; thus, the results are difficult to interpret. Despite this problem, however, the overall effect of temperature is greater than would be expected for a physical process, such as diffusion, and is consistent with the view that an energy requiring step is involved.

Since the translocation of gold particles through the pores is i) dependent on the properties of the adsorbed RNA-coat, ii) likely involved in a transport process and iii) not an artifact of the technique, it is concluded that the pathways visualized for the translocation of RNA-coated colloidal tracers are the same as those normally used for endogenous RNA. These pathways are located in the centers of the nuclear pores and have apparent functional diameters of approximately 230 Å. These results are consistent with those obtained previously by Stevens and Swift (1966) for mRNA efflux in Chironomus salivary gland cells.

It is not known whether tRNA and 5S RNA complex with specific proteins prior to exiting the nucleus. There is evidence that the

poly(A) tails of mRNA bind different polypeptides in the nucleus and cytoplasm (Baer and Kornberg, 1983; Sachs and Kornberg, 1985; Setyono and Greenberg, 1981); however, it has not been determined if these proteins are involved in transport.

The fact that over 97% of the pores are involved in RNA efflux, combined with the earlier observation that the majority of the pores contained nucleoplasmin-coated particles following cytoplasmic injections (Feldherr et al., 1984), suggests that these pathways are bifunctional. The double injection experiments provided direct evidence supporting this view. Thus, it appears that the central channels within the pores can function in the translocation of both RNA and protein. However, this does not necessarily mean that the same molecular mechanisms are employed.

Six hours after injection, the nuclear to cytoplasmic concentration ratio of RNA-coated gold was found to be 14:1. Correcting for the difference in the volumes of the two compartments, it is estimated that no more than 36% of the particles entered the cytoplasm. Based on data presented by Zasloff (1983, Table I) an equivalent amount of radiolabelled tRNA would be expected to leave the nucleus in about 3 h. There are several factors that could account for this difference. First, the conformation of tRNA could be modified after adsorption to the gold particle. In this regard, Tobian et al. (1985) found that point mutations which alter the conformation of tRNA also decrease its rate of transport to the cytoplasm. Second, since several tRNA molecules are adsorbed to the gold particles (the exact number has not been determined) the binding avidity to components within the nucleus,

pores or even the adjacent cytoplasm could be affected. Third, the size of the tracers could influence the transport rates. Although RNA-coated particles leave the nucleus at a reduced rate, it should be emphasized that the properties of RNA required for translocation through the pores are retained.

Zasloff (1983) and De Robertis et al. (1982) reported that radiolabelled tRNA is unable to enter the nucleus following injection into the cytoplasm; however, the exchange of 5S RNA across the envelope does appear to be bidirectional (De Robertis et al., 1982). Gold particles coated with tRNA, 5S RNA, or poly(A) were able to pass from the cytoplasm into the nucleus, but exchange across the envelope was greatly restricted. Even after 1 h the nuclear to cytoplasmic concentration ratio was only about 0.01. Considering these low rates of uptake, it was surprising to find that RNA-coated particles were observed extending through the centers of the pores. These results could be interpreted to mean that the translocation of RNA through the pores is a reversible process, but that release into the nucleoplasm, which could require a separate mechanism, might be a limiting factor. However, before any definitive conclusion can be drawn it will be necessary to obtain reliable quantitative data concerning the percentage of pores that contain gold and additional information relating to the nuclear uptake rates. Furthermore, it is not known whether translocation is initiated by the adsorbed RNA itself or whether the particles fortuitously bind karyophilic proteins which, in turn, induce transport (Mattaj, 1986). Hopefully it will be possible to resolve some of these questions using isolated nuclei.

CHAPTER III
THE EFFECTS OF VARIATIONS IN THE NUMBER AND SEQUENCE OF TARGETING SIGNALS ON NUCLEAR UPTAKE

Introduction

The nuclear pore complex has been identified as the major, if not the exclusive site for macromolecular diffusion and transport between the nucleus and cytoplasm of eukaryotic cells (Feldherr et al., 1962; Feldherr et al. 1984). Evidence for mediated protein transport was determined intially for RN1 (Feldherr et al., 1983) and nucleoplasmin (Dingwall et al., 1982), both of which are major karyophilic proteins found in amphibian oocytes. From electron microscopic analysis of the intracellular distribution of nucleoplasmin-coated gold particles, it was established that the transport channels, located in the centers of the pores, are at least 200 Å in diameter (Feldherr, et al., 1984). In contrast, the channel has a functional diameter of approximately 90 Å with respect to diffusion (Paine et al., 1975).

Several laboratories have utilized recombinant DNA methodology and single amino acid substitutions to obtain probes useful for the localization and characterization of transport signals that target specific proteins to the nucleus. These approaches have been used to study nuclear uptake of the following proteins; nucleoplasmin (Burglin and De Robertis, 1987; Dingwall et al., 1987), simian virus (SV 40) large T-antigen (Kalderon et al., 1984a,b), the yeast regulatory proteins MATα2 (Hall et al., 1984) and GAL4 (Silver et al., 1984), yeast

histones 2A and 2B (Moreland et al., 1987), the yeast ribosomal protein L3 (Moreland et al., 1985), polyoma large T-antigen (Richardson et al., 1986), and the adenovirus E1a protein (Lyons et al., 1987). Although there is no concensus signal, it appears that nuclear targeting is dependent on short, basic amino acid sequences.

Of the proteins listed above, SV 40 large T-antigen has been studied most extensively. Kalderon et al. (1984b) constructed hybrid proteins by linking various amino acid sequences found in SV 40 large T-antigen to the amino terminus of pyruvate kinase and found that the shortest sequence capable of targeting the enzyme to the nucleus was Pro-Lys-Lys^{128}-Lys-Arg-Lys-Val. Transport is especially sensitive to a point mutation at the Lys^{128} position (Kalderon et al., 1984a; Lanford et al., 1986). Amino acid mutations in the vicinity of the Lys^{128} position reduces, but does not necessarily abolish transport of SV 40 large T-antigen into the nucleus. Roberts et al. (1987) have shown that multiple copies of a partially defective signal can cooperate to enhance nuclear localization.

Lanford et al. (1986) synthesized peptides that contained the SV 40 large T transport signal and cross-linked them to several carrier proteins (ovalbumin, BSA, IgG, sIgA, ferritin and IgM). When the conjugates were injected into the cytoplasm of cultured cells, all but IgM (m.w. 970 kd) entered the nucleus. Qualitative evidence, utilizing indirect immunofluorescence, suggested that the number of signals per carrier protein can affect the rate of uptake. Goldfarb et al. (1986) found that BSA conjugated with SV 40 large T-antigen signals accumulates

in the nuclei of Xenopus oocytes with saturable uptake kinetics, suggesting the involvement of a receptor-mediated process.

Nucleoplasmin, a 110 kd pentameric karyophilic protein (as determined by sequence analysis), also has been used extensively to study nuclear transport in oocytes (Dingwall et al., 1982; Feldherr et al., 1984) and cultured cells (Schulz and Peters, 1986; Sugawa et al., 1985). It has recently been determined that each monomeric subunit contains 1 targeting signal. Furthermore, removal of one or more signal domains by proteolytic cleavage markedly reduces the rate of nuclear uptake (Dingwall et al., 1982). The region containing the signal has been sequenced and although it is similar to the SV 40 targeting signal, it is not identical (Dingwall, personal communication).

The data summarized above suggest that variations in the nuclear targeting signal could significantly influence nucleocytoplasmic exchange of proteins. To better understand the nature of these effects, experiments were performed to determine how macromolecules containing different signal sequences and different numbers of signals interact with, and modulate the properties of the nuclear pores. Various size colloidal gold particles were coated with 1) BSA conjugated with different numbers of synthetic peptides containing the SV 40 targeting signal, 2) BSA conjugated with inactive SV 40 signals, 3) large T-antigen, or 4) nucleoplasmin. These tracers were microinjected into oocytes and their distribution within the oocytes was later determined by electron microscopy.

The data indicate that as the number of signals per molecule increases, both the relative uptake of the tracer particles into the

nucleus and the functional size of the transport channels increase. The SV 40 and nucleoplasmin targeting sequences varied in their ability to facilitate transport. This could be related to differences in their binding affinity for nuclear envelope (pore) receptors. Double labelling experiments demonstrated that different targeting signals can be recognized by and transported through the same pore. Control experiments ruled out the possibilities of nonspecific exchange processes.

Materials and Methods

Xenopus laevis were purchased from Xenopus I (Ann Arbor, Michigan) and maintained as reported previously (Feldherr, 1975).

Colloidal gold coating agents

The synthesis of peptides containing the SV 40 large T-antigen targeting signals and their conjugation to BSA was described in detail by Lanford et al. (1986). In brief, the thirteen amino acid sequence was synthesized on a glycyl-Merrifield resin (Merrifield, 1963) using a Biosearch Sam Two (Biosearch, San Rafael, CA) automated peptide synthesizer. The synthetic peptides were conjugated to BSA using the heterobifunctional cross-linking agent m-maleimido benzoyl-N-hydroxysuccimide ester (MBS; Pierce Chemical Co., Rockford, IL). Dialysis in phosphate-buffered saline followed by repeated concentration and dilution of the conjugates separated the unconjugated peptides from the carrier proteins. By varying the molar ratios of the reactants, different protein-peptide coupling ratios were obtained. Amino acid

analyses were performed with a Beckman Model 7300 analyzer (Beckman Instruments Inc., Palo Alto, CA) to obtain a number-average ratio of signal peptides to carrier protein BSA. The peptide to carrier protein ratios obtained by SDS-PAGE were similar to those calculated by amino acid analysis.

Large T-antigen was expressed in insect cells using the baculovirus expression vector system (Luckow and Summers, 1988) and was purified by immunoaffinity chromatography as described previously (Siamins and Lane, 1985). Nucleoplasmin was isolated using an anti-nucleoplasmin IgG affinity column as described previously (see Chapter II, Materials and Methods). BSA used in the control experiments was purchased from Sigma Chemical Co. (St. Louis, MO).

The BSA conjugates used as coating agents are listed in Table VI. The table shows the synthetic 13 amino acid sequence conjugated to BSA and the average number of peptides per BSA molecule. The synthetic peptide contains the seven amino acid sequence required for nuclear localization of the SV 40 large-T antigen (Lanford et al., 1986). The conjugates denoted BSA-WT represent preparations that contain active nuclear targeting signals. In the BSA-cT conjugates, neutral asparagine was substituted for the second lysine (equivalent to Lys^{128} in SV 40 large T-antigen) in the synthetic peptide (see Table VI). The resulting signal is similar to the mutation present in the SV 40(cT)-3 mutant (Lanford and Butel, 1984), and is defective in transport. To obtain a coating agent containing an average of 3 signals per carrier molecule, the $BSA\text{-}WT_8$ conjugate was diluted 3-fold with the $BSA\text{-}cT_7$ preparation prior to stabilizing the gold particles. The use of $BSA\text{-}cT_7$ to dilute

Table VI - Protein Preparations Used as Coating Agents

Coating Agent	Average No. of Signals	Sequence Conjugated to BSA
BSA	0	
BSA-WT_5	5	Cys-Gly-Tyr-Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val-Gly-Gly
BSA-WT_8	8	
BSA-WT_{11}	11	
BSA-cT_7	7	Cys-Gly-Tyr-Gly-Pro-Lys-Asn-Lys-Arg-Lys-Val-Gly-Gly
BSA-cT_{13}	13	
BSA-WT_8 + cT_7*	3	
Large T-antigen	1 per monomer	
Nucleoplasmin	1 per monomer	

The underlined sequence represents the active (WT preparations) and inactive (cT preparations) SV 40 large T nuclear transport signal.

*The average number of signals obtained for this coating agent was performed by diluting BSA-WT_8 with BSA-cT_7.

the active signal assured that the overall properties (shape and size) of the particles were consistent with those of other tracers used in this investigation. Purified SV 40 large T-antigen, which has 1 signal per monomer, and nucleoplasmin, which has a different signal sequence, also were used as coating agents. BSA alone was employed as an additional control.

Preparation and stabilization of colloidal gold

The colloidal gold fractions that contained particles 20-50 Å and 20-160 Å in diameter were both prepared by reducing chloroauric acid with a solution of white phosphorus in ether (Feldherr, 1965). 50-280 Å gold particles were obtained by adding 2.5 ml of 0.6% gold chloride to a freshly prepared 20-160 Å fraction, 1 ml of additional reducing agent was then added, and the preparation was boiled for 2-3 minutes. Fractions containing 120-280 Å particles were prepared by reducing chloroauric acid with trisodium citrate, as described previously (Frens, 1973). The 50-280 Å and 120-280 Å gold sols, were brought to pH 7.0 with 0.72 N K_2CO_3.

Prior to stabilization, all of the coating agents were either dialyzed against, or dissolved in, a low ionic strength buffer (7.2 mM K_2HPO_4 and 4.8 mM KH_2PO_4, pH 7.0). The volumes of the different agents required to stabilize 1 ml of the gold sols are listed in Table VII. The procedure used to determine these volumes is outlined in Feldherr et al. (1984).

After stabilization, the 20-160 Å and 50-280 Å preparations were centrifuged at 2000g at 4°C for 10 minutes to remove any aggregates of

Table VII - Volumes of Coating Agent (µl) Required to Stabilize 1 ml of Gold Sol*

Coating Agents	(conc.)	Fraction Size 20-50 Å	20-160 Å	50-280 Å	120-280 Å
BSA	(1.0mg/ml)	60	--	--	--
BSA-WT_5	(1.3mg/ml)	10	60	40	--
BSA-WT_8	(0.6mg/ml)	30	150	150	20
BSA-WT_{11}	(0.5mg/ml)	--	200	--	15
BSA-cT_7	(1.4mg/ml)	20	80	--	--
BSA-cT_{13}	(0.6mg/ml)	--	--	140	--
Large T-Ag	(0.4mg/ml)	--	400	--	--
Nucleoplasmin	(0.6mg/ml)	--	40	150	25

*These are average values, intended to serve as a guide. The exact amounts of coating agents required for stabilization should be determined for each individual gold preparation.

gold. Centrifugation of the 20-50 Å and 120-280 Å fractions was not necessary. In each instance, 5-7 ml of stabilized colloid were concentrated to 70-100 µl in Minicon concentrators (Amicon Corp., Danvers, MA) and dialyzed against intracellular injection medium (102 mM KCl, 11.1 mM NaCl, 7.2 mM K_2HPO_4 and 4.8 mM KH_2PO_4, pH 7.0) at 4°C prior to injection.

Injection

Late stage 5 and stage 6 oocytes (Dumont, 1972) were manually defolliculated in amphibian Ringer's solution and centrifuged at approximately 650g for 8-10 minutes (Kressman and Birnstiel, 1980). The cells were then microinjected with approximately 40 nl of gold sol at a site adjacent to the nucleus and fixed at intervals of 15 min, 1 h, 6 or 20 h. The tip diameters of the micropipettes were 10-15 µm.

Electron microscopy and analysis

The cells were fixed for electron microscopy and prepared for sectioning as described previously (see Chapter 2, Materials and Methods). Relative nuclear uptake of gold particles stabilized with the different coating agents was determined by counting particles in equal and adjacent areas of the nucleus and cytoplasm close to the site of injection. Yolk granules and mitochondria were excluded from the analyses. The counts are reported as nuclear to cytoplasmic ratios (N/C). The size distributions of particles present within the nucleus and cytoplasm were determined by direct measurement from electron micrograph negatives. The envelope to cytoplasm ratios were obtained by

comparing the number of particles associated with the cytoplasmic surface of the envelope (i.e., at or within 650 Å of the nuclear surface) to the number of particles in an equal, randomly selected area of cytoplasm.

Negative staining with 1% phosphotungstic acid was used to estimate the thickness of the adsorbed protein coats.

By extrapolating from data published by De Roe et al. (1984) and correcting for additional mass contributed by the peptides, estimates were made of the number of BSA molecules adsorbed onto the surfaces of different size gold particles. For example, particles with diameters of 35 Å, 80 Å, 140 Å, and 180 Å (the mean size of the 4 different gold preparations injected) would have 2, 8, 24, and 39 molecules of BSA conjugates, respectively. Knowing the number of BSA molecules adsorbed and the synthetic peptide to carrier protein ratios, it was possible to estimate the total number of SV 40 large T-antigen targeting signals on the different size gold particles; however, the proportion of signal actually available for transport (i.e., exposed signals) is not known.

Results

Cytoplasmic injections of the tracer particles

It was determined initially that microinjection of approximately 40 nl of stabilized gold adjacent to the nucleus delivered a sufficient number of particles for electron microscopic analysis. The protein content of the injectate ranged from 50-300 ng depending on the size of the gold fraction and the specific coating agent used.

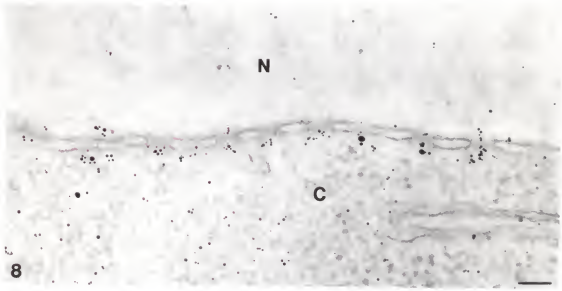
All preparations containing active transport signals (BSA-WT conjugates, large T-antigen or nucleoplasmin) were translocated into the nucleus through central channels located within the nuclear pores. In the region of injection, the particles were uniformly distributed in the cytoplasm; however, at longer time intervals, 6 and 20 h, the BSA-WT conjugates occasionally formed aggregates. The reason for this is not known, but it appeared to be dependent on the presence of active signals since similar aggregates were not observed with BSA-cT conjugates. At all time intervals, particles containing active transport signals were present both within the pores and the nucleoplasm. With increasing time there was a concomitant increase in the number of particles present in the nucleus (data not shown). Gold coated with the BSA-cT conjugates or BSA alone were essentially excluded from the pores and nucleoplasm. These general distributions are illustrated in Figs. 8 and 9, which show the results obtained 1 h after injecting particles coated with BSA-WT_{11} and BSA-cT_7, respectively.

Relative uptake

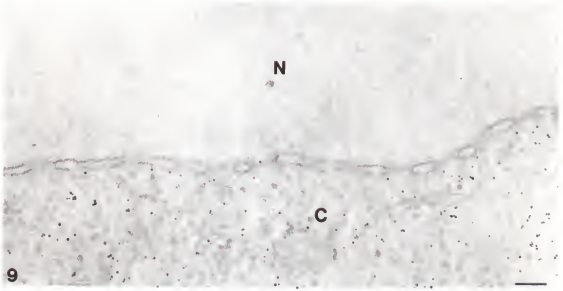
Quantitative data, dependent on the specific properties of the individual coating agents, were obtained 1 h after injection. This time interval allowed for sufficient particle distribution within the cytoplasm, and minimized possible loss or redistribution of soluble cell components caused by the injection procedures (Miller et al, 1984). The N/C ratios obtained at 1 h do not represent equilibrium values since nuclear uptake was observed to increase with time, but reflect relative

Figure 8. BSA-WT_{11}-gold. - 1 h experiment.
Gold particles (20-160 Å), near the site of injection, are observed evenly distributed throughout the cytoplasm (C). Particles are seen passing through the centers of the pores and also within the nucleus (N). Bar, 0.1 µm.

Figure 9. BSA-cT_7-gold - 1 h experiment.
Tracer particles (20-50 Å), near the site of injection, are distributed evenly throughout the cytoplasm (C), but are rarely seen within the nucleus (N) or along the envelope. Bar, 0.1 µm.



8



9

uptake of the tracer particles. Four to 6 cells were examined for each coating agent within each size fraction.

The nuclear to cytoplasmic (N/C) ratios for different coating agents are given in Table VIII. After 1 h, N/C ratios obtained with the 20-50 Å fraction stabilized with BSA-WT_5 and BSA-WT_8 were 0.58 and 0.76, respectively. The difference in these ratios, as determined by the Student's t-test, is not statistically significant ($p>0.25$). When the particle size was increased to 20-160 Å or 50-280 Å in diameter, the differences in N/C ratios between the BSA-WT_5 and BSA-WT_8 conjugates were highly significant ($p<0.002$). In addition, BSA-WT_5 was more effective in facilitating transport than large T-antigen which contains 1 signal per monomer ($p<0.002$). However, increasing the number of signals from 8 (BSA-WT_8) to 11 (BSA-WT_{11}) did not significantly increase the N/C ratio (p values obtained for the 20-160 Å and 120-280 Å fractions were $p>0.8$ and $0.1>p>0.05$, respectively).

The N/C ratio obtained for the BSA-WT_8-cT_7 dilution (approximately 3 signals per BSA molecule) was significantly lower than that obtained for the BSA-WT_5 conjugate ($p<0.002$); however, it was also significantly lower than that observed for large T-antigen-gold ($0.01<p<0.02$). This could be due to variation in the availability and/or spatial distribution of the signal at the surface of the gold particles.

The N/C values for BSA-cT_7-, BSA-cT_{13}-, and BSA-coated gold were significantly lower than all gold preparations containing active nuclear targeting signals ($p<0.002$).

Overall, it is concluded from these results that there is a direct correlation between the number of transport signals and the relative

Table VIII - N/C Ratios* 1 h

Coating Agents	20-50 Å	20-160 Å	50-280 Å	120-260 Å
BSA	0.009	----	----	----
BSA-cT_7	0.01	0.009	----	----
BSA-cT_{13}	----	----	0.006	----
BSA-WT_8 + BSA-cT_7 (1:3 dilution)	----	0.035	----	----
BSA-WT_5	0.58	0.18	0.06	----
BSA-WT_8	0.76	0.79	0.38	0.14
BSA-WT_{11}	----	0.80	----	0.24
Large T-Antigen	----	0.077	----	----
Nucleoplasmin	----	2.43	0.71	0.51

*N/C ratios were calculated from 500-3000 particle counts per data point.

uptake of particles into the nucleus. Furthermore, the data suggest that as the size of the particles increases, more signals are required for their transport across the envelope.

To compare the effectiveness of a different targeting signal, parallel studies were performed with nucleoplasmin-gold. The relative uptake of different size nucleoplasmin-coated particles by the nucleus is shown in Table VIII. The N/C ratios determined for nucleoplasmin-coated particles were significantly greater than those obtained for BSA-WT_5-, BSA-WT_8-, or BSA-WT_{11}-coated gold. In all instances, the probability values were $p<0.002$. Since there are only 5 targeting signals per nucleoplasmin molecule, these differences in uptake suggest that the nucleoplasmin transport signal is more effective than the SV 40 large T-antigen targeting sequence, at least in oocytes.

Size distributions

In view of the above results, the size distributions of particles in different regions of the oocytes were analyzed in more detail. The nuclear and cytoplasmic size distributions determined for BSA-WT_5 and BSA-WT_8 1 h after injection are given in Table IX and Fig. 10. Seventeen percent of the BSA-WT_8-coated particles that entered the nucleus were larger than 157 Å, compared to 5.6% for particles coated with BSA-WT_5. When particles larger than 185 Å are compared, the difference in uptake is almost 10-fold. The difference in the size of the particles able to penetrate the pores is statistically significant as determined by Chi-square analysis.

TABLE IX - Size Distribution of BSA-WT_5-and WT_8-coated Particles in Injected Cells*

Experiment (1 h)	Total No. of Particles Measured	Percentage of Particles in Each Size Class# 45-73 Å	73-101 Å	101-129 Å	129-157 Å	157-185 Å	185-213 Å	213-241 Å	241-269 Å	>269 Å
BSA-WT_5										
Nucleus	406	31.5	19.7	24.6	18.5	4.9	0.7	-	-	-
Cytoplasm	533	3.2	11.1	24.2	29.8	14.8	9.0	3.8	1.1	2.8
BSA-WT_8										
Nucleus	1018	14.3	20.3	24.6	23.8	10.1	4.8	1.7	0.4	-
Cytoplasm	808	5.2	13.1	25.0	28.2	14.7	7.4	3.6	1.7	0.9

*These experiments were performed with 45-280 Å gold particles and the mean size of the fraction was 140 Å.

#The size of the gold particles does not include the thickness of the coating agent.

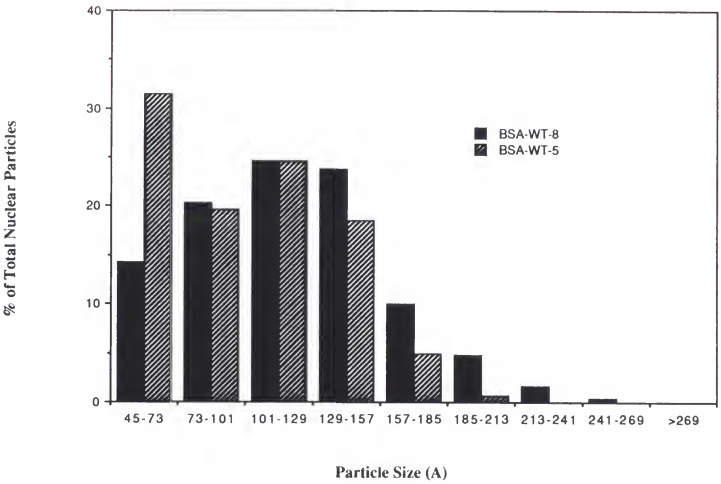


Figure 10. Nuclear particle distributions: BSA-WT_8 and BSA-WT_5

When the number of signals per BSA molecule is increased beyond 8 there is no further increase in functional pore size. This is indicated in Fig. 11, which compares the size distribution of BSA-WT_8-, BSA-WT_{11}- and nucleoplasmin-coated particles within the nucleus 1 h after injecting a 120-280 Å gold fraction. The sizes of the particles able to pass through the pores did not vary significantly for the different coating agents. A comparison of the cytoplasmic distributions to the nuclear distributions, as shown in Table X, demonstrates that particles larger than 230 Å (average of the size class), do not readily penetrate the pores, regardless of the coating agent. These results demonstrate that the maximum size particle able to enter the nucleus is approximately 260 Å in diameter. This value includes the thickness of the coat material, which adds about 30 Å to the overall particle diameter.

In contrast, the large T-antigen data given in Table XI indicate that particles larger than 90 Å were not detected in the nucleus after 1 h. The size data obtained for the BSA-WT_8-cT_7 dilutional experiment (Table XI) gave similar results, although a few particles larger than 90 Å were present in the nucleus.

Overall, analysis of the size distributions of particles in the nucleus and cytoplasm indicate a direct relationship between the functional dimensions of nuclear pores and the number of active SV 40 large T targeting signals per BSA molecule.

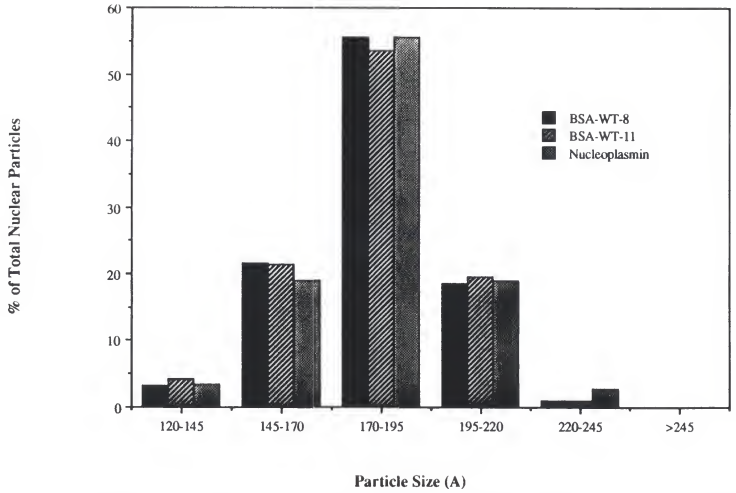


Figure 11. Nuclear particle distributions: BSA-WT_8, BSA-WT_{11}, and nucleoplasmin

TABLE X - Size Distribution of BSA-WT_8-, WT_{11}- and Nucleoplasmin-coated Particles in Injected Cells*

Experiment (1 h)	Total No. of Particles Measured	Percentage of Particles in Each Size Class# 120-145 Å	145-170 Å	170-195 Å	195-220 Å	220-245 Å	>245 Å
BSA-WT_8							
Nucleus	371	3.2	21.6	55.5	18.6	1.1	--
Cytoplasm	556	2.9	16.5	51.3	21.9	5.9	1.4
BSA-WT_{11}							
Nucleus	358	4.2	21.5	53.6	19.6	1.1	--
Cytoplasm	557	3.2	14.7	50.6	22.8	7.0	1.6
Nucleoplasmin							
Nucleus	514	3.5	18.9	55.6	19.0	2.4	--
Cytoplasm	525	3.2	16.4	49.3	20.4	7.2	3.4

*These experiments were performed with 120-260 Å gold particles and the mean size of the fraction was 180 Å.

#The size of the gold particles does not include the thickness of the coating agent.

TABLE XI - Size Distribution of BSA-WT_8-cT_7- and Large T-Ag-coated Particles in Injected Cells*

Experiment (1 h)	Total No. of Particles Measured	Percentage of Particles in Each Size Class# 10-30 Å	30-50 Å	50-70 Å	70-90 Å	90-110 Å	>110 Å
Large T-Ag							
Nucleus	386	22.8	49.0	26.2	2.0	--	--
Cytoplasm	600	16.2	30.3	39.3	8.8	3.7	1.7
BSA-WT_8-cT_7 (1:3 dilution)							
Nucleus	574	26.0	40.1	27.4	5.2	0.7	0.2
Cytoplasm	621	11.6	23.7	37.6	16.7	7.1	3.6

*These experiments were performed with 20-120 Å gold particles and the mean size of the fraction was 60 Å.

#The size of the gold particles does not include the thickness of the coating agent.

Accumulation of tracers along the nuclear envelope

Although the nuclear size distributions were the same for particles coated with the BSA conjugates (WT_8 or WT_{11}) or nucleoplasmin, there was a significant difference in the relative uptake of the gold as a function of the coating agent, indicating that not all transport signals are equally effective. The results shown in Fig. 12 and Table XII suggest that the effectiveness of specific signals could be related to their ability to bind to the nuclear envelope.

Figure 12 illustrates the intracellular distributions of 50-280 Å gold particles coated with BSA-WT_5 (12a), BSA-WT_8 (12b) and nucleoplasmin (12c). Similar amounts of colloid were injected into each oocyte. Progressing from 12a to 12c, there is an increase in the number of particles present both within the nucleus and along the envelope. The relationship between the accumulation of particles along the envelope and relative uptake is shown in Table XII. The envelope to cytoplasm ratios were obtained by comparing the number of particles associated with the envelope to the number of particles in an equal, randomly selected area of cytoplasm. It is evident that a direct relationship exists between the number of particles associated with the envelope and nuclear uptake. A similar relationship between uptake and binding was obtained with the 120-280 Å particle fractions, coated with BSA-WT_8, BSA-WT_{11}, or nucleoplasmin (data not shown).

Coinjection of BSA-WT_8- and nucleoplasmin-coated particles

To determine if different targeting signals can be transported through the same pore, small particles (20-50 Å), coated with BSA-WT_8,

Figure 12. Accumulation of tracers along the nuclear envelope - 1 h experiment.
A comparison of the intracellular distributions of 50-280 Å gold particles coated with BSA-WT_5 (12a), BSA-WT_8 (12b) and nucleoplasmin (12c). Similar amounts of colloid were injected in each oocyte. Progressing from a to c, there is an increase in the number of particles present both within the nucleus (N) and along the nuclear envelope. C, cytoplasm. Bar, 0.2 μm.

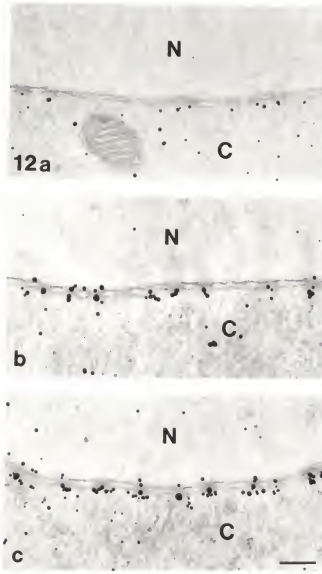

N
C
12a
N
C
b
N
C
c

Table XII - Envelope-Associated Particles

Coating Agent	Envelope:cytoplasm Ratios*	N/C Ratio
BSA-WT_5	1.2	0.06
BSA-WT_8	2.7	0.35
Nucleoplasmin	6.3	0.71

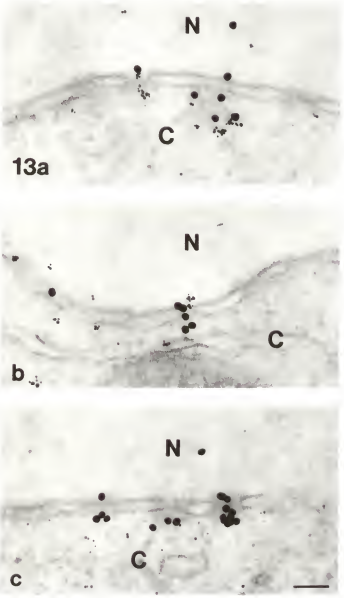
*These ratios were obtained 1 h after injection as described in the text.

and large particles (120-280 Å), coated with nucleoplasmin, were injected simultaneously into the cytoplasm. As seen in Figs. 13a and 13b, small particles and large particles are located either within or adjacent to the nuclear surface of the same nuclear pore. To control for nonspecific binding of the small particles to the large particles, or possible exchange of coat material during the injection procedure, experiments were also performed in which small gold particles were coated with BSA-cT_7 and mixed with the large nucleoplasmin-gold particles prior to injection. Electron microscopic analysis of this experiment, shown in Fig. 13c, indicates that only large particles are present in the pores and the nucleus while the small tracer particles are retained in the cytoplasm. These experiments demonstrate that individual pores can recognize and translocate different nuclear targeting signals.

Discussion

By stabilizing colloidal gold particles with BSA conjugated with synthetic peptides that contained either active or inactive SV 40 large T-antigen nuclear transport signals, it was possible to prepare a range of electron microscopic tracers that varied in both size and signal content. Electron microscopic analysis of the intracellular distributions of the tracer particles following microinjection into oocytes led to the following conclusions. First, BSA conjugates containing active signals are transported into the nucleus through central channels located within the pores. Second, both the functional size of the channels and relative nuclear uptake increase as the signal

Figure 13. Coinjection of gold particles coated with BSA-conjugates and nucleoplasmin - 1 h experiments.
13a and 13b, large nucleoplasmin-coated particles (120-280 Å) and small BSA-WT_8-coated particles (20-50 Å) can both be seen within the pore region or just at the nuclear surface of the pores. 13c, large nucleoplasmin-coated particles are seen entering the nucleus (N) while small BSA-cT_7-coated particles are retained in the cytoplasm (C).
Bar, 0.1 µm.


N
C
13a
N
C
b
N
C
c

number per gold particle increases. Furthermore, the maximum size of the transport channel is estimated to be 260 Å in diameter. Control experiments, utilizing BSA conjugated with inactive signals, demonstrated that differences in the uptake of gold particles coated with different BSA-WT conjugates were due to variations in the number of active signals and not to nonspecific factors such as alterations in particle size and charge.

Nucleoplasmin-coated gold was used as a stabilizing agent to compare the effectiveness of a different nuclear targeting signal. It was found that nucleoplasmin, BSA-WT_8, and BSA-WT_{11} all had similar effects on the functional size of the transport channel, even though nucleoplasmin has fewer signals than either of the conjugates. However, the relative uptake of nucleoplasmin-coated gold was significantly greater than that observed for particles coated with the BSA-WT conjugates. The fact that nucleoplasmin-gold accumulated along the nuclear surface to a greater degree than other tracers suggests that the relative effectiveness of different targeting sequences might be related to their binding affinity for transport receptors.

The possibility that binding might be an important step in the transport process was originally suggested by Feldherr et al. (1984), and was based on the observation that nucleoplasmin-gold particles accumulate at the surface of the pores during translocation. Other data in support of this view are the kinetic studies by Goldfarb et al. (1986) which demonstrate that the nuclear uptake of BSA conjugated with large T targeting signals is saturable and, therefore, likely to be a receptor-mediated process. Furthermore, Newmeyer and Forbes (1988)

obtained evidence that transport involves two separate events; the first is binding to the pores, which is signal sequence dependent, and the second is translocation into the nucleus, which is ATP dependent. Overall, the data obtained in this study are consistent with the view that transport occurs through the pores by a gated process. I would suggest that a 90 Å channel is normally present within the pores allowing for the diffusion of smaller macromolecules into and out of the nucleus (Paine, 1975). However, in response to an appropriate transport signals, the dimensions of the channel can increase in size to accommodate the uptake of transportable (nondiffusive) macromolecules.

The results obtained with nucleoplasmin and the BSA conjugates indicate that the extent of channel dilation might be variable and dependent on the number of simultaneous interactions between signals and receptors. Thus, the degree to which the channels are dilated is likely to be modulated by a combination of two factors, 1) the number of transport signals available and 2) the binding affinity of the signals for the receptors. According to this model, a small number of high affinity signals might be as effective in regulating the size of the transport channel as a larger number of low affinity signals.

In evaluating the effect of signal number on the translocation process, it should be kept in mind that endogenous karyophilic proteins would also contribute to the total pool of transport signals. The degree to which endogenous proteins might influence the uptake of the tracer particles cannot be determined at this time.

Coinjection of different size gold particles, coated with proteins containing different nuclear targeting signals (BSA-WT and

nucleoplasmin), demonstrated that individual pores are capable of recognizing and transporting different nuclear targeting signals. In a study to show the bidirectional capability of the nuclear pores, it was demonstrated that individual pores can transport both protein and RNA (Chapter II, Results). Whether all transport signals can be recognized by each pore has yet to be determined.

CHAPTER IV
SUMMARY AND PROSPECTUS

Summary of Results

In the present study, microinjection of colloidal gold particles coated with different RNAs or proteins was used to establish morphological criteria concerning the nucleocytoplasmic exchange of macromolecules *in vivo*. The results from the injection of RNA-coated particles (Chapter II) can be summarized as follows: first, central channels located within the nuclear pores are visualized as the major, if not exclusive, site for RNA translocation into the cytoplasm. Second, poly(A)-coated particles, at least 230 Å in diameter, can penetrate the envelope. Third, most, if not all, of the nuclear pores have the ability to transport the RNA tracer particles. Finally, individual pores can be bifunctional, i.e., they can recognize and transport both RNA and protein. The results presented in chapter III have established how variations in the number and amino acid sequence of protein transport signals can affect protein uptake. First, it was found that particles coated with BSA conjugated with synthetic peptides that contained the SV 40 large T-antigen transport signal translocate into the nucleus through the centers of the nuclear pores. It was then demonstrated that variations in the number and sequence of protein transport signals can affect both the functional size of the transport channel and the relative uptake of particles into the nucleus. The

maximum functional size of the transport channel was estimated to be 260 Å in diameter. Furthermore, it was found that individual pores can recognize and transport proteins containing different targeting signals. The ramifications of these results are discussed in relation to how the pores might regulate nucleocytoplasmic exchange and thus, be intimately involved in the modulation of cellular activity.

The selectivity of the nucleocytoplasmic exchange process can occur at the level of the nuclear envelope, within the nucleus or cytoplasm. Since the nuclear pores represent the major sites of exchange for macromolecules, their role in regulating the exchange process is of special interest.

Nuclear Envelope Selectivity

As discussed in Chapter I, the patent size of the nuclear pore can determine the size of the molecule which can diffuse into and out of the nucleus, with the rate of uptake or efflux being inversely related to size. This mode of selection by the envelope can be considered one level of specificity since any molecule that is larger than the diffusion channel (90-120 Å in diameter) tends to be excluded from the nucleus, unless a specific transport process is involved.

Several lines of evidence have clearly established that a selection process exists for the transport of proteins into the nucleus. It has been demonstrated for a number of nuclear proteins (Chapter I, endogeneous macromolecules), that information which specifies selective nuclear entry resides in a region of their primary sequence. Presumably, the interaction of the targeting signal with the cytoplasmic

surface of the nuclear pore most likely involves the specific recognition of the signal by a pore receptor. This interaction provides the cell with an additional level of control since binding allows for the selection of transportable molecules. The removal or alteration of these targeting signals by either proteolytic cleavage or DNA methodology results in the protein retaining its cytoplasmic location.

The results in Chapters II and III indicate that translocation of gold particles coated with RNA, nucleoplasmin, or the BSA-WT-conjugates occurs through the centers of the pores and not along the periphery which indicates the involvement of common pathways. The translocation of the tracers was found to be a selective process. In all cases, particles coated with synthetic polymers (PVP or polyglutamic acid) or exogenous macromolecules (BSA or ovalbumin) were virtually excluded from the pores, demonstrating that the RNA- and BSA-WT-coated gold translocation is due to the presence of the coating agent. In addition, the size of the particles, up to 260 Å in diameter, that penetrate the pore region far exceeds the upper limit of diffusion, suggesting that an active transport process is involved.

It was demonstrated that most, if not all, of the nuclear pores are capable of transporting different classes of RNA. Similar results were obtained after cytoplasmic injection of gold particles coated with proteins containing active targeting signals. Visualization of the nuclear envelope in the region of injection indicated that a large percentage of the pores contained protein-coated gold particles. In addition, the double injection experiments showed that individual pores are capable of translocating both RNA-coated and protein-coated gold

particles and they can also recognize and transport proteins containing different nuclear targeting signals. Based upon these results it is suggested that nuclear pores have a broad range of specificity and distinct functional classes of pores might be nonexistent.

Since the signals from large T-antigen cross-linked to BSA and different RNAs are capable of translocating through the same nuclear pores as nucleoplasmin, it is of interest whether they all use the same mechanism for translocation. It is not known if the RNAs complex with proteins which subsequently initiate their transport or whether RNA itself has a putative transport signal. Since BSA-WT- and nucleoplasmin-coated particles can translocate through the same pore it remains to be determined if there are different receptors for different transport signals within individual pores. Although a concensus sequence for protein transport signals does not exist, signal sequences usually contain a short stretch of basic amino acids. In this respect, nucleoplasmin and large T-antigen share partial homology.

It is clear that specificity observed for the nucleocytoplasmic exchange of endogeneous macromolecules cannot be accounted for entirely by the properties of the pores. Thus, it is likely that selectivity of proteins and RNA designated for transport can also occur within either the nuclear or cytoplasmic compartments. For example, mRNA must be released from the intranuclear matrix with the hydrolysis of ATP prior to efflux (Schroder et al., 1987). The presence of poly(A) tails on mRNA might be part of the selection process; however, poly(A)$^-$mRNA also must somehow be selected since histone mRNA is not polyadenylated. In addition, many of the proteins can be bound within the cytoplasm or

nucleus, thus being denied access to the pores and preventing transport. Therefore, the cell can have molecules with active transport signals; however, they may be inaccessible for transport due to selective binding away from the pores or specific masking of the signal.

Dynamic Aspects of the Transport Channels

In addition to localizing the pathway for exchange, microinjection of the tracers provided a method for analyzing the morphological and functional characteristics of the transport channel. After injection of a gold fraction ranging from 50-280 Å in diameter, the largest particles capable of penetrating the pores were 230 Å. When including the size of the coat material (approximately 30 Å), the maximum functional size of the transport channel was estimated to be 260 Å in diameter. Since it is unlikely that a single endogenous protein would have as high a number of transport signals as the BSA-WT_8 and BSA-WT_{11} conjugates, 8 and 11 signals respectively, it is of interest to consider the possible biological significance for a channel of this size. First, it is likely that several endogenous molecules are continually being transported into and out of the nucleus and simultaneous exchange through the pores might require a full dilation of the transport channel. A second requirement for the 260 Å translocation channel is the transport of large ribosomal components and mRNPs that are synthesized in the nucleus and pass through the pores into the cytoplasm. Stevens and Swift (1966) and Skoglund et al. (1983) have shown that a large mRNP particle, approximately 260 Å in diameter, passes through the nuclear pores of

Chironomus salivary gland cells. These results provide direct evidence for the requirement of a 260 Å transport channel.

Two possible mechanisms were explored to determine how the cell and the pore complex might regulate the exchange of proteins across the nuclear envelope. Since the number of synthetic peptides per carrier protein could be altered and the SV 40 large T-antigen signal was not homologous to the nucleoplasmin transport signal, the effect of variations in signal number and sequence on protein transport through the pores was studied.

At the surface of the pore, the interaction between the signal and its receptor sets up another point of regulatory control by the nuclear envelope. As shown in chapter III, the functional size of the transport channel and relative uptake is dependent on both the nature of the signal and number of targeting signals per molecule. The results indicate a direct relationship between signal number and the size of the tracer particles that can penetrate the nuclear pores. The functional size of the channel would be dependent on the total number of available signals accessible to the pore at a given time.

In addition to signal number, the affinity of the signal for its receptor could affect the uptake of coated particles. A comparison of uptake and accumulation of tracers at the nuclear surface suggests that the nucleoplasmin signal has a higher affinity for the pore receptors than the large T-antigen signal. This could explain why nucleoplasmin-coated gold, which has a fewer number of signals than BSA-WT_8 and BSA-WT_{11}, is transported more efficiently than the active BSA-conjugates. Lanford et al., (1988) studied the effects of amino acid substitutions

within the synthetic peptide signal on protein transport. They found the rate of transport decreased when different basic amino acids were substituted in the lysine 128 position, and that transport activity was abolished when neutral asparagine was used. Based on these findings, I would propose that a change of amino acids within the signal sequence can alter the affinity of the signal for the receptor, resulting in slower uptake.

Proposed Model

The transport channel is not a fixed structure since it can fluctuate between 90 Å (patent diffusion channel) and 260 Å (maximum size of the transport channel) in diameter, the functional diameter being dependent on the number of binding events between receptors and transport signals. There appear to be at least two factors involved in the variability of the transport channel: the signal number and affinity. From this data, I would propose that the mechanism for protein transport through the nuclear pores of oocytes occurs by a gated process. Normally, in an unactivated state, the channel within the pore would be 90 Å in diameter and available for diffusion into and out of the nucleus. However, activation by transport signals would result in the dilation of the transport channel to an extent which is directly related to the number of active signals bound to the receptors at or within the pore complex. Furthermore, a small number of high affinity binding signals might be as effective in regulating the size of the transport channel as a larger number of low affinity signals. Alternatively, the dilation of the pore might be an all or none process

rather than a channel opening incrementally. Although the protein uptake data favors the latter, the two mechanisms cannot be distinguished at this time.

Consistent with the gating model are the results presented by Newmeyer and Forbes (1988) and Richardson et al. (1988). They show that the transport of proteins across the envelope in vitro can be experimentally separated into at least two steps. The first step is the binding of the proteins to the cytoplasmic pore surface which involves the specific recognition of the transport signal by the nuclear pore complex. Binding to this region is signal sequence dependent, thus again demonstrating selectivity by the pore complex, and ATP independent. The second step is the ATP dependent translocation through the pores.

According to the model, multiple interactions need occur prior to distention of the transport channel. If so, then multiple copies of receptors should reside in the pore complex. Components speculated to be part of the translocation machinery are the group of glycoproteins localized to the pore complex by Snow et al. (1987), Finlay et al. (1967), and Davis and Blobel (1986). Although these glycoproteins have not been demonstrated to function as receptors, they are present in multiple copies within each pore complex, consistent with the gating model. Furthermore, in an attempt to define the role of the glycoproteins in protein transport it was shown that WGA binds to the pore region and inhibits the uptake of nucleoplasmin in synthetic nuclei (Finlay et al., 1987) and cultured cells (Yoneda et al., 1987), but does not block the diffusion channel.

There are several implications of the model with regard to regulation of nucleocytoplasmic exchange. The dilation of the pores would control exchange in accordance to the related needs of the cell and provide the cell with an efficient method for exchange. In addition, the model suggests that endogenous molecules available for transport can act collectively to open the transport channels. The factors involved in regulating pore activation, signal number and affinity, are supportive of this model. To increase the rate of uptake, a single protein could have multiple signals (i.e., nucleoplasmin) or proteins might aggregate to increase their overall signal number, possibly like histones. Furthermore, proteins containing different targeting signals might have differential binding affinities for pore receptors which, in turn, might modulate their rate of uptake. Finally, the complete activation of the pores is large enough for the transfer of ribosomal subunits and RNP particles.

Future Trends

One question that remains is whether the protein transport signal is vectorial. In a cursory study, nuclear injection of the protein conjugates was performed to determine if the signal for protein uptake is bidirectional, i.e., capable of transport into and out of the nucleus. One and six hours after nuclear injection, particles coated with the BSA-conjugates were not observed in either the pores or the cytoplasm (data not presented) indicating that transport signal is vectorial; however, the experiments are not definitive. It is possible that the gold particles were bound specifically or nonspecifically

within the nucleus due to the signals or the BSA, thus making the gold conjugates inaccessible to the pores. Interestingly, the signal region of large T-antigen is not the same as the DNA binding domain and preliminary results suggest that BSA alone is not concentrated within the nucleus; thus, the signal might be vectorial in nature. One possible reason for the nuclear retention of the conjugates is that the signal becomes masked upon nuclear entry which then causes the efflux process to be inactive. The best evidence for the masking or unmasking of transport signals is the snRNA associated proteins binding to snRNAs to form snRNPs prior to nuclear entry (Mattaj and De Robertis, 1985). Although the complex may be diffusable within its cellular compartment, masking of the transport signal can be considered one form of binding that blocks bidirectional transport.

Another explanation to support vectorial transport is the asymmetric distribution of transport receptors. Snow et al. (1987) have indicated that one of their antibodies, specific for a glycoprotein, labels almost exclusively the nucleoplasmic side of the pore complex. Although this glycoprotein is not known to function as a receptor, its asymmetric distribution is consistent with the view of vectorial transport. Until more is known about the intranuclear interactions of the tracer particles, it cannot be concluded if the uptake signals are unidirectional. The use of resealed nuclear envelope vesicles (see chapter I), which contain no intranuclear components, could be one method for studying this question.

Additional mechanisms that the cell could use to regulate exchange of macromolecules across the envelope include: i) changing the

permeability properties of the nuclear pores and/or ii) altering the number of pores per envelope. It has been shown in amoebae that the nuclear pores have some variability with respect to diffusion that occurs during different stages of the cell cycle (Feldherr, 1966). In addition, it was demonstrated that there are changes in the functional properties of the pores during different metabolic states of the amoebae and the differences in uptake were not due to a change in pore number or size (Feldherr, 1971).

Maul et al. (1972) have demonstrated that pore formation in HeLa cells during the cell cycle is biphasic. They found two significant increases in pore formation during mitosis, the first being within an hour after division and the second, shortly after the beginning of S phase. Furthermore, they found that the number of pores per nucleus can vary with a change in cellular activity. Thus, the cell, in principle, can regulate the rate of accumulation by changing the number of pores available for nucleocytoplasmic exchange.

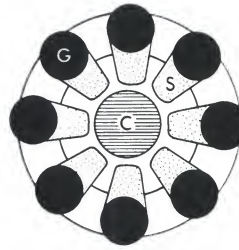
In contrast to diffusion (see above), a question that remains to be investigated is whether the transport properties of the pores change significantly during the cell cycle and/or during changes in cellular activity. Dreyer et al. (1985) have suggested that the temporal accumulation of different proteins can be due to the nature and number of the transport signals. However, as mentioned above, pore variability can be dependent on the physiological state of the cell. Since the proteins do not undergo posttranslational modification and they distribute differently at various times throughout development, they might be responding to changes in the envelope. At present, it is not

known if the nuclear envelope transport properties are altered during changes in cellular activities. These results can also be explained by de novo synthesis of specific carriers which subsequently result in a shift in a molecule's partitioning between cytoplasm and nucleus although no carriers for nuclear transport have been identified.

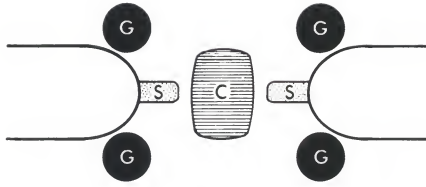
What is the underlying molecular mechanism required for the movement of macromolecules through the length of the transport channel? Berrios et al. (1983) have identified an ATPase polypeptide associated with nuclear-envelope enriched fractions. The molecular mechanisms for the actual translocation has been postulated to be a contractile force provided by this ATPase activity which is a myosin-like heavy chain polypeptide localized in the nuclear periphery (Berrios and Fischer, 1986). However, it remains to be determined if the ATPase activity localizes predominantly in the pore complexes. Schindler and Jiang (1986) used the fluorescence redistribution after photobleaching technique to study factors that influence the flux rates of dextrans across the nuclear envelope of isolated nuclei. They found that the addition of anti-actin antibodies markedly reduced the rate of nuclear entry of the labelled dextran. Furthermore, anti-myosin antibodies significantly blocked the ATP stimulatory effects on the dextran flux rate. From these results, it was postulated that the contractile proteins, actin and myosin, are part of the pore complex and involved in the opening and closing of the pores. The problem is that these results have not been verified *in vivo*, and it is known that nuclear isolation can significantly alter the properties of the pores.

Isolation of receptors involved in the translocation process remains an essential step to understanding the nucleocytoplasmic transport of macromolecules. Additionally, it is equally important to determine if there are different receptors in individual pores or whether there are common receptors for the transport of different protein signal sequences and/or putative RNA transport signals. After identification of receptors an important area that remains to be investigated is the binding affinities of different transport signals as the affinity of signals is most likely, in part, a mechanism that the cell can use to regulate nucleocytoplasmic exchanges.

APPENDIX



Top View



Side View

A schematic representation of the nuclear pore complex based on data from Franke's (1974) and Unwin and Milligan's (1982) model. G, annular globules; S, spokes; C, central granule.

REFERENCES

Aaronsen, R.P., and G. Blobel. 1975. Isolation of nuclear pore complexes in association with a lamina. Proc. Natl. Acad. Sci. USA 72:1007-1011.

Abelson, P.H., and W.R. Duryee. 1949. Radioactive sodium permeability and exchange in frog eggs. Biol. Bul. 96:205-217.

Agutter, P.S. 1985a. RNA processing, RNA transport and nuclear structure. In Nuclear Envelope Structure and RNA Maturation. UCLA Symposia on Molecular and Cellular Biology. E.A. Smuckler and G.A. Clawson, editors. Vol. 26. Alan R. Liss, Inc., New York. 539-559.

Agutter, P.S. 1985b. Nuclear Envelope NTPase and RNA efflux. In Nuclear Envelope Structure and RNA Maturation. UCLA Symposia on Molecular and Cellular Biology. E.A. Smuckler and G.A. Clawson, editors. Vol. 26. Alan R. Liss, Inc., New York. 561-578.

Agutter, P.S., J.R. Harris, and I. Stevenson. 1977. RNA stimulation of mammalian liver nuclear envelope nucleoside triphosphatase. A possible marker for the nuclear envelope. Biochem. J. 162:671-679.

Agutter, P., H. McArdle, and B. McCaldin. 1976. Evidence for the involvement of nuclear envelope NTPase in nucleocytoplasmic translocation of ribonucleoprotein. Nature (Lond.) 263:165-167.

Agutter, P.S., B. McCaldin, and H.J. McArdle. 1979. Importance of mammalian nuclear-envelope nucleoside triphosphatase in nucleocytoplasmic transport of ribonucleoproteins. Biochem. J. 182:811-819.

Austerberry, C.F., and P.L. Paine. 1982. In vivo distribution of proteins within a single cell. Clin. Chem. 28:1011-1014.

Baer, B., and R.D. Kornberg. 1983. The protein responsible for the repeating structure of cytoplasmic poly(A)-ribonucleoprotein. J. Cell Biol. 96:717-721.

Berezney, R. 1980. Fractionation of the nuclear matrix. I. Partial separation into matrix protein fibrils and a residual ribonucleoprotein fraction. J. Cell Biol. 85:641-650.

Bernd, A., H.C. Schroder, R.K. Zahn, and W.E.G. Muller. 1982. Modulation of the nuclear-envelope nucleoside triphosphatase by poly(A)-rich mRNA and by microtubule protein. Eur. J. Biochem. 129:43-49.

Berrios, M., G. Blobel, and P.A. Fischer. 1983. Characterization of an ATPase/dATPase activity associated with the Drosophila nuclear matrix-pore complex-lamina fraction. Identification of the putative enzyme polypeptide by direct ultraviolet photoaffinity labeling. J. Biol. Chem. 258:4548-4555.

Berrios, M., and P.A. Fisher. 1986. A myosin heavy chain-like polypeptide is associated with the nuclear envelope in higher eukaryotic cells. J. Cell Biol. 103:711-724.

Bonner, W.M. 1975a. Protein migration into nuclei. I. Frog oocyte nuclei in vivo accumulate microinjected histones, allow entry to small proteins, and exclude large proteins. J. Cell Biol. 64:421-430.

Bonner, W.M. 1975b. Protein migration into nuclei. II. Frog oocyte nuclei accumulate a class of microinjected oocyte nuclear proteins and exclude a class of microinjected oocyte cytoplasmic proteins. J. Cell Biol. 64:431-437.

Burglin, T.R., and E.M. De Robertis. 1987. The nuclear migration signal of Xenopus laevis nucleoplasmin. EMBO J. 6:2617-2625.

Butel, J.S., M.J. Guentzel, and F. Rapp. 1969. Variants of defective simian papovirus 40 (PARA) characterized by cytoplasmic localization of simian papovirus 40 tumor antigen. J. Virol. 4:632-641.

Callan, H.G., and S.G. Tomlin. 1950. Experimental studies on amphibian oocyte nuclei. I. Investigation of the structure of the nuclear membrane by means of the electron microscope. Proc. Roy. Soc. London B137:367-378.

Century, T.J., I.R. Fenichel, and S.B. Horowitz. 1970. The concentrations of water, sodium and potassium in the nucleus and cytoplasm of amphibian oocytes. J. Cell Sci. 7:5-13.

Chambers, R., and B. Fell. 1931. Micro-operations on cells in tissue cultures. Proc. Roy. Soc. London. B109:380-403.

Clawson, G.A., C.M. Feldherr, and E.A. Smuckler. 1985. Nucleocytoplasmic RNA transport. Mol. Cell. Biochem. 67:87-99.

Clawson, G.A., D.S. Friend, and E.A. Smuckler. 1984. Localization of nucleoside triphosphatase activity to the inner nuclear envelope and associated heterochromatin. Exp. Cell Res. 155:310-314.

Clawson, G.A., J. James, C.H. Woo, D.S. Friend, D. Moody, and E.A. Smuckler. 1980. Pertinence of nuclear envelope nucleoside triphosphatase activity to ribonucleic acid transport. Biochem. 19:2748-2756.

Clawson, G.A., M. Koplitz, B. Castler-Schechter, and E.A.Smuckler. 1978. Energy utilization and RNA transport: Their interdependence. Biochem. 17:3747-3752.

Clawson, G.A., M. Koplitz, D.E. Moody, and E.A. Smuckler. 1980. Effects of thioacetamide treatment on nuclear envelope nucleoside triphosphatase activity and transport of RNA from rat liver nuclei. Cancer Res. 40:75-79.

Clawson, G.A., C.H. Woo, J. Button, and E.A. Smuckler. 1984. Photoaffinity labeling of the major nucleosidetriphosphatase of rat liver nuclear envelope. Biochem. 23:3501-3507.

Davis, L.I., and G. Blobel. 1986. Identification and characterization of a nuclear pore complex protein. Cell 45:699-709.

De Robertis, E.M., S. Lienhard, and R.F. Parisot. 1982. Intracellular transport of microinjected 5S and small nuclear RNAs. Nature 295:572-577.

De Robertis, E.M., R.F. Longthorne, and J.B. Gurdon. 1978. Intracellular migration of nuclear proteins in Xenopus oocytes. Nature 272:254-256.

De Roe, C., P.J. Courtoy, J. Quintart, and P. Baudhuin. 1984. Molecular aspects of the interactions between proteins and colloidal gold. J. Cell Biol. 99:57a.

Diberardino, M.A., N.J. Hoffner, and M.B. Matilsky. 1977. Methods of studying nucleocytoplasmic exchange of nonhistone proteins in embryos. In Methods in Cell Biology. Chromatin and Chromosomal Protein Research. D.M. Prescott, editor. Vol. XVI. Academic Press, New York, 141-165.

Dingwall, C., S.M. Dilworth, S.J. Black, S.E. Kearsey, L.S. Cox, and R.A. Laskey. 1987. Nucleoplasmin cDNA sequence reveals polyglutamic acid tracts and a cluster of sequences homologous to putative nuclear localization signals. EMBO J. 6:69-74.

Dingwall, C., and R.A. Laskey. 1986. Protein import into the cell nucleus. Ann. Rev. Cell Biol. 2:367-90.

Dingwall, C., S.V. Sharnick, and R.A. Laskey. 1982. A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. Cell 30:449-458.

Dreyer, C., R. Stick, and P. Hausen. 1986. Uptake of oocyte nuclear proteins by nuclei of Xenopus embryos. In Nucleocytoplasmic Transport. R. Peters and M. Trendelenburg, editors. Springer-Verlag, Berlin. 143-157.

Dumont, J.N. 1972. Oogenesis in Xenopus laevis (Daudin). I. Stages of oocyte development in laboratory maintained animals. J. Morphol. 136:153-179.

Dwyer, N., and G. Blobel. 1976. A modified procedure for the isolation of a pore complex-lamina fraction from rat liver nuclei. J. Cell Biol. 70:581-591.

Earnshaw, W.C., B.M. Honda, R.A. Laskey, and O.J. Thomas. 1980. Assembly of nucleosomes: the reaction involving X. laevis nucleoplasmin. Cell 21:373-383.

Feldherr, C.M. 1962. The nuclear annuli as pathways for nucleo-cytoplasmic exchanges. J. Cell Biol. 14:65-72.

Feldherr, C.M. 1965. The effect of the electron-opaque pore material on exchanges through the nuclear annuli. J. Cell Biol. 25:43-53.

Feldherr, C.M. 1966. Nucleocytoplasmic exchanges during cell division. J. Cell Biol. 31:199-203.

Feldherr, C.M. 1971. Evidence for changes in nuclear permeability during different physiological states. Tissue and Cell 3:1-8.

Feldherr, C.M. 1975. The uptake of endogenous proteins by oocyte nuclei. Exp. Cell Res. 93:411-419.

Feldherr, C.M. 1980. Ribosomal RNA synthesis and transport following disruption of the nuclear envelope. Cell Tissue Res. 205:157-162.

Feldherr, C.M., R.J. Cohen, and J.A. Ogburn. 1983. Evidence for mediated protein uptake by amphibian oocyte nuclei. J. Cell Biol. 96:1486-1490.

Feldherr, C.M., E. Kallenbach, and N. Schultz. 1984. Movement of a karyophilic protein through the nuclear pores of oocytes. J. Cell Biol. 99:2216-2222.

Feldherr, C.M., and J.A. Ogburn. 1980. Mechanism for the selection of nuclear polypeptides in Xenopus oocytes. II. Two-dimensional gel analysis. J. Cell Biol. 87:589-593.

Feldherr, C.M., and J. Pomerantz. 1978. Mechanism for the selection of nuclear polypeptides in Xenopus oocytes. J. Cell Biol. 78:168-175.

Fey, E.G., D.A. Ornelles, and S. Penman. 1986. Association of RNA with the cytoskeleton and the nuclear matrix. J. Cell Sci. 5(Suppl.):99-119.

Finlay, D.R., D.D. Newmeyer, T.M. Price, and D.J. Forbes. 1987. Inhibition of in vitro nuclear transport by a lectin that binds to nuclear pores. J. Cell Biol. 104:189-200.

Forbes, D.J., M.W. Kirschner, and J.W. Newport. 1983. Spontaneous formation of nucleus-like structures around bacteriophage DNA microinjected into eggs. Cell 34:13-23.

Ford, P.J. 1971. Non-coordinated accumulation and synthesis of 5S ribonucleic acid by ovaries of Xenopus laevis. Nature (Lond.) 233:561-564.

Frank, M., and S.B. Horowitz. 1975. Nucleocytoplasmic transport and distribution of an amino acid, in situ. J. Cell Sci. 19:127-139.

Franke, W.W. 1966. Isolated nuclear membranes. J. Cell Biol. 31: 619-623.

Franke, W.W. 1974. Structure, biochemistry, and functions of the nuclear envelope. Int. Rev. Cytol. Suppl. 4:71-236.

Franke, W.W., and H. Falk. 1970. Appearance of nuclear pore complexes after Bernhard's staining procedure. Histochemie 24:266-278.

Franke, W.W., and U. Scheer. 1974. Pathways of nucleocytoplasmic translocation of ribonucleoproteins. Symp. Soc. Exp. Biol. XXVIII:249-282.

Frens, G. 1973. Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. Nature Physical Science 241:20-22.

Gerace, L., and G. Blobel. 1982. Nuclear lamina and the structural organization of the nuclear envelope. Cold Spring Harbor Symp. Quant. Biol. 46:967-978.

Gerace, L.C., A. Blum, and G. Blobel. 1978. Immunocytochemical localization of the major polypeptides of the nuclear pores complex-lamina fraction. Interphase and mitotic distribution. J. Cell Biol. 79:546-566.

Gerace, L., C. Comeau, and M. Benson. 1984. Organization and modulation of nuclear lamina structure. J. Cell Sci. Suppl. 1:137-160.

Gerace, L., Y. Ottaviano, and C. Kondor-Koch. 1982. Identification of a major polypeptide of the nuclear pore. J. Cell Biol. 95:826-837.

Giese, G., and F. Wunderlich. 1983. In vitro ribosomal ribonucleoprotein transport. Temperature-induced 'graded unlocking' of nuclei. J. Biol. Chem. 131-135.

Goldfarb, D.S., J. Gariepy, G. Schoolnik, and R.D. Kornberg. 1986. Synthetic peptides as nuclear localization signals. Nature 322:641-644.

Goldstein, L., and W. Plaut. 1955. Direct evidence for nuclear synthesis of cytoplasmic ribose nucleic acid. Proc. Natl. Acad. Sci. 41:874-880.

Gurdon, J.B. 1970. Nuclear transplantation and the control of gene activity in animal development. Proc. Roy. Soc. Lond. B176:303-314.

Hall, M.N., L. Hereford, and I. Herskowitz. 1984. Targeting of E. coli β-galactosidase to the nucleus in yeast. Cell 36:1057-1065.

Harris, J.R. 1978. The biochemistry and ultrastructure of the nuclear envelope. Biochem. Biophy. Acta. 515:55-104.

Horowitz, S.B. 1972. The permeability of the amphibian oocyte nucleus, in situ. J. Cell Biol. 54:609-625.

Horowitz, S.B., and I.R. Fenichel. 1970. Analysis of sodium transport in the amphibian oocyte by extractive and radioautographic techniques. J. Cell Biol. 47:120-131.

Horowitz, S.B., and L.C. Moore. 1974. The nuclear permeability, intracellular distribution, and diffusion of inulin in the amphibian oocyte. J. Cell Biol. 60:405-415.

Jeffery, W.R. 1982. Messenger RNA in the cytoskeletal framework: Analysis by in situ hybridization. J. Cell Biol. 95:1-7.

Kalderon, D., W.D. Richardson, A.F. Markham, and A.E. Smith. 1984a. Sequence requirements for nuclear localization of simian virus 40 large-T antigen. Nature 311:33-38.

Kalderon, D., B.L. Roberts, W.D. Richardson, and A.E. Smith. 1984b. A short amino acid sequence able to specify nuclear location. Cell 39:499-509.

Kalt, M.R., and B. Tandler. 1971. A study of fixation of early amphibian embryos for electron microscopy. J. Ultrastruct. Res. 36:633-645.

Kanno, Y., and W.R. Loewenstein. 1963. A study of the nucleus and cell membrane of oocytes with an intra-cellular electrode. Exp. Cell Res. 31:149-166.

Kim, S.D., G.J. Quigley, F.L. Suddath, A. McPherson, D. Sneden, J.J. Kim, J. Weinzierl, and A. Rich. 1973. Three-dimensional structure of yeast phenylalanine transfer RNA; Folding of the polynucleotide chain. Science (Wash.) 179:285-288.

Kleinschmidt, J.A., C. Dingwall, G. Maier, and W.W. Franke. 1986. Molecular characterization of a karyophilic, histone-binding protein: cDNA cloning, amino acid sequence and expression of nuclear proteins N1/N2 of Xenopus laevis. EMBO J. 5:3547-3552.

Kohen, E., G. Siebert, and C. Kohen. 1971. Transfer of metabolites across the nuclear membrane: A microfluorometric study. Hoppe-Seyler's Z. Physiol. Chem. 352:927-937.

Kressman, A., and M.L. Birnstiel. 1980. Surrogate genetics in the frog oocyte. In Transfer of Cell Constituents into Eukaryotic Cells. J.E. Celis, A. Graessmann, and A Loyter, editors. Plenum Press, New York. 383-407.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 227:680-685.

Lam, K.S, and C.B. Kasper. 1979. Selective phosphorylation of a nuclear envelope polypeptide by an endogenous protein kinase. Biochem. 18:307-311.

Lanford, R.E., and J.S. Butel. 1980. Biochemical characterization of nuclear and cytoplasmic forms of SV 40 tumor antigens encoded by parental and transport-defective mutant SV40-adenovirus T hybrid viruses. Virology 105:314-327.

Lanford, R.E., and J.S. Butel. 1984. Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. Cell 37:801-813.

Lanford, R.E., P.. Kanda, and R.C. Kennedy. 1986. Induction of nuclear transport with a synthetic peptide homologous to the SV40 T antigen transport signal. Cell 46:575-582.

Lanford, R.E., R.G. White, R.G. Dunham, and P. Kanda. Effects of basic and nonbasic amino acid substitutions on transport induced by SV40 T antigen synthetic peptide nuclear transport signals. (Submitted for publication).

Luckow, V.A., and M.D. Summers. 1988. Trends in the development of bacolovirus expression vectors. Bio/Technology 6:47-55.

Lyons, R.H., B.Q. Ferguson, and M. Rosenberg. 1987. Pentapeptide nuclear localization signal in adenovirus E1a. Mol. Cell. Biol. 7:2451-2456.

Mattaj, I.W. 1986. The role of RNA-Protein interactions in intracellular targeting. In Nucleocytoplasmic Transport. R. Peters and M. Trendelenburg, editors. Springer-Verlag, Berlin. 275-285.

Mattaj, I.W., and E.M. De Robertis. 1985. Nuclear segregation of U2 snRNA requires binding of specific snRNP proteins. Cell 40:111-118.

Mattaj, I.W., S. Lienhard, R. Zeller, and E.M. De Robertis. 1983. Nuclear exclusion of transcription factor IIIA and the 42S particle transfer RNA-binding protein in Xenopus oocytes: A possible mechanism for gene control? J. Cell Biol. 97:1261-1265.

Maul, G.G. 1977. The nuclear and the cytoplasmic pore complex: Structure, dynamics, distribution, and evolution. Int. Rev. Cytol. Suppl. 6:75-186.

Maul, G.G., and F.A. Baglia. 1983. Localization of a major nuclear envelope protein by differential solubilization. Exp. Cell Res. 145:285-292.

Maul, G.G., H.M. Maul, J.E. Scogna, M.W. Lieberman, G.S. Stein, B.Y. Hsu, and T.W. Borun. Time sequence of nuclear pore formation in phytohemagglutinin-stimulated lymphocytes and in HeLa cells during the cell cycle. J. Cell Biol. 55:433-447.

McDonald, J.R., and P.S. Agutter. 1980. The relationship between polyribonucleotide binding and the phosphorylation and dephosphorylation of nuclear envelope protein. FEBS Lett. 116:145-148.

Melton, D.A., E.M. De Robertis, and R. Cortese. 1980. Order and intracellular location of the events involved in the maturation of a spliced tRNA. Nature 284:143-148.

Merrifield, R.B. 1963. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J. Am. Chem. Soc. 85:2149-2154.

Miller, D.S., Y.T. Lau, and S.B. Horowitz. 1984. Artifacts caused by cell microinjection. Proc. Natl. Acad. Sci. USA 81:1426-1430.

Mills, A.D., R.A. Laskey, P. Black, and E.M. De Robertis. 1980. An acidic protein which assembles nucleosomes in vitro is the most abundant protein in Xenopus oocyte nuclei. J. Mol. Biol. 139:561-568.

Moffett, R.B., and T.E. Webb. 1981. Regulated transport of messenger ribonucleic acid from isolated liver nuclei by nucleic acid binding proteins. Biochem. 20:3253-3262.

Moffett, R.B., and T.E. Webb. 1983. Characterization of a messenger transport protein. Biochem. Biophys. Acta 740:231-242.

Moreland, R.B., G.L. Langevin, R.H. Singer, R.L. Garcea, and L.M. Hereford. 1987. Amino acid sequences that determine the nuclear localization of yeast histone 2B. Mol. Cell. Biol. 7:4048-4057.

Moreland, R.B., H.G. Nam, L.M. Hereford, and H.M. Fried. 1985. Identification of a nuclear localization signal of a yeast ribosomal protein. Proc. Natl. Acad. Sci. USA 82:6561-6565.

Murty, C.N., E. Verney, and H. Sidransky. 1980. Effect of tryptophan on nuclear envelope nucleoside triphosphatase in rat liver. Proc. Soc. Exp. Biol. Med. 163:155-161.

Naora, H., H. Naora, M. Izawa, V.G. Allfrey, and A.E. Mirsky. 1962. Some observations on differences in composition between the nucleus and cytoplasm of the frog oocyte. Proc. Natl. Acad. Sci. USA 48:853-859.

Newmeyer, D.D., and D.J. Forbes. 1988. Nuclear import can be separated into two distinct steps in vitro: Nuclear pore binding and translocation. Cell 52:641-653.

Newmeyer, D.D., J.M. Lucocq, T.R. Burglin, and E.M. De Robertis. 1986. Assembly in vitro of nuclei active in nuclear protein transport: ATP is required for nucleoplasmin accumulation. EMBO J. 5:501-510.

Newport, J., and M. Kirschner. 1982. A major developmental transition in early Xenopus embryos: I. Characterization and timing of cellular changes at the midblastula stage. Cell 30:675-686.

Paine, P.L. 1975. Nucleocytoplasmic movement of fluorescent tracers microinjected into living salivary gland cells. J. Cell Biol. 66:652-657.

Paine, P.L., C.F. Austerberry, L.J. Desjarlais, and S.B. Horowitz. 1983. Protein loss during nuclear isolation. J. Cell Biol. 97:1240-1242.

Paine, P.L., and C.M. Feldherr. 1972. Nucleocytoplasmic exchange of macromolecules. Exp. Cell Res. 74:81-98.

Paine, P.L., and S.B. Horowitz. 1980. The movement of material between nucleus and cytoplasm. In Cell Biology. D.M. Prescott and L. Goldstein, editors. Vol. 4. Academic Press, New York. 299-338.

Paine, P.L., L.C. Moore, and S.B. Horowitz. 1975. Nuclear envelope permeability. Nature (Lond.) 254:109-114.

Pelham, H.R.B., and D.D. Brown. 1980. A specific transcription factor that can bind either the 5S RNA gene or 5S RNA. Proc. Natl. Acad. Sci. USA 77:4170-4174.

Peters, R. 1983. Nuclear envelope permeability measured by fluorescence microphotolysis of single liver cell nuclei. J. Cell Biol. 258:11427-11429.

Peters, R. 1984. Nucleo-cytoplasmic flux and intracellular mobility in single hepatocytes measured by fluorescence microphotolysis. EMBO J. 3:1831-1836.

Peters, R. 1986. Fluorescence microphotolysis to measure nucleocytoplasmic transport and intracellular mobility. Biochim. Biophys. Acta. 864:305-359.

Picard, B., and M. Wegnez. 1979. Isolation of a 7S particle from Xenopus laevis oocytes: A 5S RNA-protein complex. Proc. Natl. Acad. Sci. USA 76:241-245.

Purrello, F., R. Vigneri, G.A. Clawson, and I.D. Goldfine. 1982. Insulin stimulation of nucleoside triphosphatase activity in isolated nuclear envelopes. Science 216:1005-1007.

Richardson, W.D., A.D. Mills, S.M. Dilworth, R.A. Laskey, and C. Dingwall. 1988. Nuclear protein migration involves two steps: Rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. Cell 52:655-664.

Richardson, W.D., B.L. Roberts, and A.E. Smith. 1986. Nuclear location signals in polyoma virus large-T. Cell 44:77-85.

Riedel, N., and H. Fasold. 1987. Preparation and characterization of nuclear-envelope vesicles from rat liver nuclei. Biochem. J. 241:203-212.

Roberts, B.L., W.D. Richardson, and A.E. Smith. 1987. The effect of protein context on nuclear location signal function. Cell 50:465-475.

Sachs, A.B., and R.D. Kornberg. 1985. Nuclear polyadenylate-binding protein. Mol. Cell. Biol. 5:1993-1996.

Schatz, G., and R.A. Butow. 1983. How are proteins imported into mitochondria? Cell 32:316-318.

Scheer, U. 1970. The ultrastructure of the nuclear envelope of amphibian oocytes: A reinvestigation. III. Actinomycin-induced decrease in central granules within the pores. J. Cell Biol. 45: 445-449.

Schindler, M., and L.W. Jiang. 1986. Nuclear actin and myosin as control elements in nucleocytoplasmic transport. J. Cell Biol. 102:859-862.

Schroder, H.C., M. Bachmann, B. Diehl-Seifert, and W.E.G. Muller. 1988. Transport of mRNA from nucleus to cytoplasm. Progress in Nuc. Acid Res. Mol. Biol. 34:89-142.

Schroder, H.C., M. Rottmann, M. Bachmann, and W.E.G. Muller. 1986a. Purification and characterization of the major nucleoside triphosphatase from rat liver nuclear envelopes. J. Biol. Chem. 261:663-668.

Schroder, H.C., M. Rottmann, M. Bachmann, W.E.G. Muller, A.R. McDonald, and P.S. Agutter. 1986b. Proteins from rat liver cytosol which stimulate mRNA transport: Purification and interactions with the nuclear envelope mRNA translocation system. Eur. J. Biochem. 159:51-59.

Schroder, H.C., D. Trolltsch, U. Friese, M. Bachmann, and W.E.G. Muller. 1987. Mature mRNA is selectively released from the nuclear matrix by an ATP/dATP-dependent mechanism sensitive to topoisomerase inhibitors. J. Biol. Chem. 262:8917-8925.

Schulz, B., and R. Peters. 1986. Intracellular transport of a karyophilic protein. In Nucleocytoplasmic Transport. R. Peters and M. Trendelenburg, editors. Springer-Verlag, Berlin. 171-184.

Setyono, B., and J.R. Greenberg. 1981. Proteins associated with poly(A) and other regions of mRNA and hnRNA molecules as investigated by crosslinking. Cell 24:775-783.

Siamins, V., and D.P. Lane. 1985. An immunoaffinity purification procedure for SV 40 large T-antigen. Virology 144:88-100.

Silver, P.A., L.P. Keegan, and M. Ptashne. 1984. Amino terminus of the yeast GAL4 gene product is sufficient for nuclear localization. Proc. Natl. Acad. Sci. USA 81:5951-5955.

Skoglund, U., K. Andersson, B. Bjorkroth, M.M. Lamb, and B. Daneholt. 1983. Visualization of the formation and transport of a specific hnRNP particle. Cell 34:847-855.

Smith, C.D., and W.W. Wells. 1983. Phosphorylation of rat liver nuclear envelopes. I. Characterization of in vitro phosphorylation. J. Biol. Chem. 258:9360-9367.

Snow, C.M., A. Senior, and L. Gerace. 1987. Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. J. Cell Biol. 104:1143-1156.

Stacey, D.W., and V.G. Allfrey. 1984. Microinjection studies of protein transit across the nuclear envelope of human cells. Exp. Cell Res. 154:283-292.

Steer, R.C., M.J. Wilson, and K. Ahmed. 1979a. Protein phosphokinase activity of rat liver nuclear membrane. Exp. Cell Res. 119:403-406.

Steer, R.C., M.J. Wilson, and K. Ahmed. 1979b. Phosphoprotein phosphatase activity of rat liver nuclear membrane. Biochem. Biophys. Res. Commun. 89:1082-1087.

Stevens, B.J., and H. Swift. 1966. RNA transport from nucleus to cytoplasm in Chironomus salivary glands. J. Cell Biol. 31:55-77.

Stuart, S.E., G.A. Clawson, F.M. Rottmann, and R.J. Patterson. 1977. RNA transport in isolated myeloma nuclei: Transport from membrane-denuded nuclei. J. Cell Biol. 72:57-66.

Sugawa, H., N. Imamoto, M. Wataya-Kaneda, and T. Uchida. 1985. Foreign protein can be carried into the nucleus of mammalian cell by conjugation with nucleoplasmin. Exp. Cell Res. 159:419-429.

Tobian, J.A., L. Drinkard, and M. Zasloff. 1985. tRNA nuclear transport: Defining the critical regions of human $tRNA_i^{met}$ by point mutagenesis. Cell 43:415-422.

Unwin, P.N.T., and R.A. Milligan. 1982. A large particle associated with the perimeter of the nuclear pore complex. J. Cell Biol. 93:63-75.

van Eekelen, C.A.G, and W.J. van Venrooij. 1981. hnRNA and its attachment to a nuclear protein matrix. J. Cell Biol. 88:554-563.

Warren, P., and B. Dobberstein. 1978. Protein transfer across microsomal membranes reassembled from separated membrane components. Nature (Lond.) 273:569-571.

Watson, M.L. 1955. The nuclear envelope: Its structure and relation to cytoplasmic membranes. J. Biophysic. Biochem. Cytol. 1:257-279.

Watson, M.L. 1959. Further observations on the nuclear envelope of the animal cell. J. Biophysic. Biochem. Cytol. 6:147-171.

Wischnitzer, S. 1958. An electron microscope study of the nuclear envelope of amphibian oocytes. J. Ultrastr. Res. 1:201-222.

Wunderlich, F., G. Giese, and H. Falk. 1983. In vitro nuclear transport of ribosomal ribonucleoprotein: Temperature affects quantity but not quality of exported particles. Mol. and Cell. Biol. 3:693-698.

Wychowski, C., D. Benichou, and M. Girard. 1986. A domain of SV40 capsid polypeptide VP1 that specifies migration into the cell nucleus. EMBO J. 5:2569-2576.

Yoneda, Y., T. Ariuka, N. Imamoto-Sonobe, H. Sugawa, Y. Shimonishi, and T. Uchida. 1987a. Synthetic peptides containing a region of SV40 large T-antigen involved in nuclear localization direct the transport of proteins into the nucleus. Exp. Cell Res. 170:439-452.

Yoneda, Y., N. Imamoto-Sonobe, M. Yamaizumi, and T. Uchida. 1987b. Reversible inhibition of protein import into the nucleus by wheat germ agglutinin injected into cultured cells. Exp. Cell Res. 173:586-595.

Zasloff, M. 1983. tRNA transport from the nucleus in a eukaryotic cell: Carrier-mediated translocation process. Proc. Natl. Acad. Sci. USA 80:6436-6440.

Zeller, R., T. Nyffenegger, and E.M. De Robertis. 1983. Nucleocytoplasmic distribution of snRNPs and stockpiled snRNA-binding proteins during oogenesis and early development in Xenopus laevis. Cell 32:425-434.

BIOGRAPHICAL SKETCH

Steven I. Dworetzky was born December 19, 1959 in New Rochelle, New York. He graduated from high school in 1977. Following high school, he attended Skidmore College in Saratoga Springs, New York, and earned a Bachelor of Arts degree in biology/chemistry in 1981. In September 1981 he entered graduate school at the University of Florida in the Department of Anatomy. In the summer of 1984, he successfully completed an eight week course entitled Cell Physiology: Cellular and Molecular Biology at the Marine Biological Laboratory in Woods Hole, Massachusetts. He completed the requirements for the degree of Doctor of Philosophy in April 1988 and accepted a postdoctoral research position at the University of Massachusetts Medical Center in Shrewsbury, Massachusetts. He is a member of the American Society for Cell Biology.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Carl M. Feldherr, Chair
Professor of Anatomy and Cell Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Robert J. Cohen
Associate Professor of Biochemistry and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Christopher M. West
Associate Professor of Anatomy and Cell Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Gary S. Stein
Professor of Biochemistry and Molecular Biology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1988

Dean, College of Medicine

Dean, Graduate School